AD_____

AWARD NUMBER:
CDMRPL-16-0-GW150051P1

TITLE: Stress Hormone Enhancement of OP-induced Neuroinflammation as an Animal Model of GWI: The Role of Toll-like Receptors and Plasticity

PRINCIPAL INVESTIGATOR: James P. O'Callaghan, Ph.D.

REPORT DATE: January 2020

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command

Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
January 2020	Final	31 Oct 2016– 30 Sep 2019

4. TITLE AND SUBTITLE
Stress Hormone Enhancement of OP-Induced Neuroinflammation as an Animal Model of GWI: The Role of Toll-like Receptors and Plasticity

5a. CONTRACT NUMBER

5b. GRANT NUMBER
CDMRPL-16-0-GW150051P1

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Lindsay T. Michalovicz, Ph.D.; Kimberly A. Kelly, Ph.D.;
James P. O'Callaghan, Ph.D.

E-Mail: jdo5@cdc.gov

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
CDC/NIOSH
1095 Willowdale Road
Morgantown, WV 26505

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

GWI is a multi-symptom disorder with features similar to "sickness behavior" (e.g., fatigue, depression, cognitive impairments, sleep disturbances, gastrointestinal problems). The exposures and conditions in theater that caused GWI remain unknown but several classes of chemicals and physiological/environmental conditions have been implicated. We are working to expand upon our previously developed mouse model of GWI combining corticosterone (CORT), as a stressor mimic, and DFP, a sarin surrogate. As such, we have tested the combination of chlorpyrifos oxon (CPO) with CORT in our long-term exposure paradigm. While the lower dose of CPO being employed in the behavioral experiments did not result in significant neuroinflammation, we have found significant alterations in Toll-like receptor signaling, as well as with the long-term DFP paradigm. Additionally, dose response studies have been performed to determine an appropriate dose for dichlorvos. In year 3, we will continue working with the CORT+CPO and CORT+DDVP regimens in the GWI model.

15. SUBJECT TERMS
Gulf War Illness, chronic neuroinflammation, chlorpyrifos oxon, physiological stress, lipopolysaccharide, dichlorvos

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified		USAMRMC

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

	Page No.
1. Introduction	4
2. Keywords	4
3. Accomplishments	4-16
4. Impact	16-17
5. Changes/Problems	17-18
6. Products	18-19
7. Participants & Other Collaborating Organizations	19-20
8. Special Reporting Requirements	20
9. Appendices	21-44

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

GWI is a multi-symptom disorder with features similar to "sickness behavior" (e.g., fatigue, depression, cognitive impairments, sleep disturbances, gastrointestinal problems). The exposures and conditions in theater that caused GWI remain unknown but several classes of chemicals and physiological/environmental conditions have been implicated. We reasoned that assessing the CNS neuroinflammatory responses known to underlie "sickness behavior," and casting a wide net for potential GW exposures and conditions, would offer the best strategy for developing an animal model of GWI. The purpose of our research is to expand on our established mouse model of GWI that combines chronic exposure to the stress hormone, corticosterone (CORT), to mimic physiological stress experienced in theater with exposure to the organophosphate and sarin surrogate, diisopropyl fluorophosphate (DFP). The neuroinflammatory effects observed after CORT + DFP exposure consisted of increased elaboration of proinflammatory cytokines and chemokines, providing the underlying molecular basis for "sickness behavior". As GW soldiers were undoubtedly exposed to a number of organophosphate agents, we have aimed to evaluate the role of other GW-relevant organophosphate compounds (e.g. chlorpyrifos and dichlorvos pesticides) in producing the neuroinflammation we have found in our GWI model. Furthermore, we aim to investigate the basis of the CORT "priming" effect, specifically evaluating the role of toll-like receptor signaling in the neuroinflammation associated with our GWI model. By identifying multiple agents that could produce GWI, we can better understand how the illness has developed and identify common neuroinflammatory mechanisms (i.e. toll-like receptor signaling) instigated by these exposures to manipulate pharmacologically.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Gulf War Illness, chronic neuroinflammation, chlorpyrifos oxon, dichlorvos, physiological stress, corticosterone priming, inflammatory mediators, gene expression

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?

The major goals of the project were: to obtain animal protocol approvals from the CDC-NIOSH ACUC and ACURO; evaluate OP-induced neuroinflammation, acetylcholinesterase activity, and "sickness behavior" with and without CORT in both a short-term and long-term exposure paradigm using the OPs: diisopropyl fluorophosphate (DFP), chlorpyrifos oxon (CPO), dichlorovs (DDVP), and physostigmine (PHY); evaluate TLR2 signaling in the short-term and long-term exposure paradigms; evaluate potential TLR2-based pharmacological interventions for GWI treatment.

Research-Specific Tasks:

	Years	% Complete
Major Task 1: Obtain Protocol Approvals		
Subtask 1: Obtain CDC-NIOSH ACUC approval for animal use in the proposed project.	1	90%
Subtask 2: Once NIOSH ACUC approval is obtained, ACURO approval for animal protocols will be completed.	1	90%
Milestone(s) Approved Protocols	1	90%
Specific Aim 1: Assess involvement of additional GWI-relevant OPs in our established CORT-primed DFP exposure GWI mouse model.		
Major Task 2: Evaluate OP-induced neuroinflammation with and without CORT		
Subtask 1: Expose mice to chronic CORT treatment for 7 days followed by DFP, CPF, DDPV, or PHY exposure on day 8. Sacrifice 6 hours after OP exposure.	1	100%
Subtask 2: Evaluate neuroinflammatory cytokines and acetylcholinesterase activity	1	100%
Subtask 3: Evaluate "sickness" behavior in Subtask 1 conditions	1-2	
Milestone(s) Achieved: Confirm expansion of the GWI phenotype beyond DFP to other irreversible acetylcholinesterase inhibitors CPF and DDPV.	1-2	100%
Milestone(s) Achieved: Prepare manuscript for publication	1-2	75%
Subtask 5: Expose mice to chronic CORT treatment for 7 days followed by DFP, CPF, DDPV, or PHY exposure on day 8. Continue CORT (7 day) treatments every other week for 90 or 180 days. At days 90 or 180 challenge mice with LPS (Sacrifice at 6 hours)	1-2	50%
Subtask 6: Evaluate neuroinflammatory cytokines.	2	50%
Subtask 7: Evaluate "sickness" behavior in Subtask 5 conditions	2	
Milestone(s) Achieved: Determine persistence of the GWI phenotype with OP irreversible AChE inhibitors.	2	50%
Milestone(s) Achieved: Prepare manuscript for publication	2	25%

Specific Aim 2: Examine the role of TLR2 signaling in neuroinflammatory responses to exposure to GWI-relevant OPs in the CORT-primed mouse model		
Major Task 3: Determine the role of the TLR2 pathway in GWI etiology		
Subtask 1: Expose mice to chronic CORT (7 days) treatment followed by OP exposure on day 8. Sacrifice 6 hours after OP exposure	2	100%
Subtask 2: Expose mice to chronic CORT (7 days) treatment followed by OP exposure on day 8. Continue CORT (7 day) treatments every other week for 90 or 180 days. At day 90 or 180, challenge mice with LPS (sacrifice at 6 hours)	2-3	50%
Subtask 3: Measure components of the TLR2 pathway at 8 and 90 or 180 days.	2-3	75%
Milestone: Determine the contribution of TLR2 pathway components in the initial priming event and persistence of the GWI phenotype.	2-3	75%
Milestone: Prepare manuscript for publication	3	
Major Task 4: Evaluate the potential for pharmacologic intervention aimed at TLR2 signaling to ameliorate neuroinflammation in GWI phenotype		
Subtask 1: Expose mice to chronic CORT (7 day) treatment followed by OP exposure on day 8. Continue CORT (7 day) treatments every other week for 90 or 180 days. At day 90 or 180 pharmacologically treat mice with minocycline 1 hour prior to LPS challenge. Sacrifice 6 hours after LPS.	2-3	100%
Subtask 2: Expose mice to chronic CORT (7 day) treatment followed by OP exposure on day 8. Continue CORT (7 day) treatments every other week for 90 or 180 days. At day 90 or 180 pharmacologically treat mice with myd88 inhibitor 1 hour prior to LPS challenge. Sacrifice 6 hours after LPS.	2-3	25%
Subtask 3: measure neuroinflammatory cytokines	3	65%
Milestone: Determine effectiveness of pharmacologic treatment to reduce LPS challenge-induced neuroinflammation	3	50%
Milestone: Prepare manuscript for publication	3	
Specific Aim 3: Define the effects of CORT-induced priming on cognitive function at the molecular and behavioral levels		

Major Task 5: Determine the effects in hippocampus of CORT + CPO on cellular BrdU labeling at 1 week and 90 days after CPO exposure		
Subtask 1: Assess the effects of CORT + CPO on cellular proliferation 1 day after BrdU administration	1	Lasley
Subtask 2: Assess the effects of CORT + CPO on cell survival 28 days after BrdU administration	1	Lasley
Subtask 3: Validate the BrdU cell counts by labeling additional tissue sections with doublecortin or NeuN	1	Lasley
Milestone: Establish the role of CORT + CPO on neurogenesis at two times after CPO	1	
Milestone: Prepare manuscript for publication	1	
Major Task 6: Evaluate the effects of CORT + CPO on expression of plasticity genes in hippocampal tissue of mice monitored for field potential responses to complex patterns of stimulation		
Subtask 1: Quantify expression of plasticity genes – *Arc, $bdnf_{total}$, $bdnf_{exonIV}$, GluA1, GluA2, NMDA epsilon 1 and 2* - in hippocampal tissue of mice monitored for synaptic transmission	1-2	Lasley 100%
Subtask 2: Record hippocampal field potential responses to single pulse, paired-pulse, and train-induced stimulation	1-2	Lasley
Milestone: Define the effect of CORT + CPO on stimulated gene expression in mice monitored for paired-pulse responses and LTP magnitude		
Major Task 7: Determine the effects of CORT + CPO on spatial learning in the Barnes maze 1 week and 90 days after CPO administration		
Subtask 1: Monitor acquisition of the maze task and probe trial performance at two time points after CORT + CPO exposure	2-3	Lasley
Milestone: Confirm the effects of GW agent exposure on behavioral performance in parallel with measures of neurogenesis, gene expression, and synaptic transmission		
Milestone: Prepare manuscript for publication		
Major Task 8: Define the efficacy of fluoxetine and a TrkB receptor agonist (DHF) in reversing the effects of CORT + CPO on synaptic transmission		

Subtask 1: Assess the trophic effects of fluoxetine (single daily dose, 5 days/week, for 4 weeks, beginning day 62) on synaptic transmission at 90 days after CORT + CPO administration	3	Lasley
Subtask 2: Assess the trophic effects of DHF (single daily dose, 5 days/week, for 4 weeks, beginning day 62) on synaptic transmission at 90 days after CORT + CPO exposure	3	Lasley
Milestones: Confirm the effectiveness of therapeutic approaches using trophic agents in ameliorating the CNS signs of CORT + CPO, i.e., GWI		
Milestone: Establish the basis for novel combination therapy to treat GWI: administration of a non-conventional anti-inflammatory agent with a CNS trophic drug		
Milestone: Prepare manuscript for publication		

What was accomplished under these goals?

Previously Reported:

An animal protocol (GW150051P1) was approved 09-12-2016 by CDC-NIOSH and ACURO for the work funded through this project. We have completed studies evaluating the "short-term" GWI exposure paradigm for DFP, CPO, and PHY and the results have been published in: Locker A.R., Michalovicz L.T., Kelly K.A., Miller J.V., Miller D.B., O'Callaghan J.P. (2017) Corticosterone primes the neuroinflammatory response to Gulf War-relevant organophosphates independently of acetylcholinesterase inhibition. *J. Neurochem.* 142(3): 444-455. In these studies, mice were exposed to corticosterone in the drinking water followed by a single exposure to one of the OPs. Both CORT+DFP and CORT+CPO resulted in significant neuroinflammation, while PHY (a reversible acetylcholinesterase inhibitor and "negative control") did not. Furthermore, it was found that while DFP, CPO, and PHY can significantly inhibit acetylcholinesterase activity, prior exposure to CORT recovered some of the inhibition instigated by DFP and CPO. This indicates a potential for high physiological stress to have protected against the lethal effects of sarin (modeled by DFP) exposure while instigating a prolonged illness in GWI.

This study has been expanded on to look at acetylcholine levels in the brain following these exposure conditions. Brain region-specific responses were found for ACh levels after DFP and CORT+DFP, resulting in amelioration of DFP-induced ACh increase when primed with CORT in the striatum and hippocampus, but not cortex (Figure 1A). This was also expanded to CPO and PHY (Figure 1B,C), however, significant amelioration of ACh increase due to CORT priming was not found in our acute mouse model. In comparison to corresponding neuroinflammatory responses, these ACh results indicate an ACh-independent mechanism for GWI-related neuroinflammation. The results from these experiments are in preparation for publication.

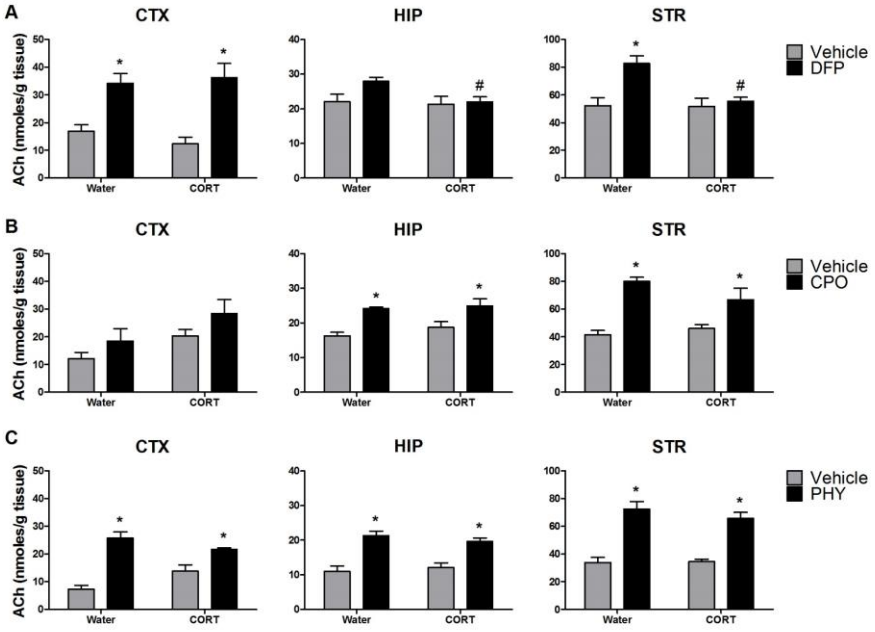

*Figure 1. ACh in discrete brain regions after A) irreversible AChEI DFP, B) irreversible AChEI CPO or C) reversible AChEI PHY exposure. ACh was measured using our HILIC-UPLC-MS/MS method developed at CDC. Mice were exposed to control or CORT (400mg/L in 1.2% EtOH) in the drinking water for 4 d. On the 5th day, mice were exposed to a single i.p. injection of peanut oil (for CPO controls), saline (for DFP, PHY controls), DFP (4 mg/kg), CPO (8 mg/kg), or PHY (0.5 mg/kg) and sacrificed via focused microwave irradiation 30 min post-DFP and CPO and 45 min post-PHY. Whole brain samples were free-hand dissected for CTX, HIP, and STR. Significance was determined using two-way ANOVA with Bonferroni post-test, where * indicates p < .05 for vehicle vs AChEI or CORT vs CORT+AChEI*

Long-term GWI paradigm studies are underway. We have found that a 5 week GWI exposure paradigm results in a significantly exacerbated neuroinflammatory response to DFP (Figure 2). In this model, the LPS challenge serves to mimic inflammatory challenges that veterans with GWI experience as "flare-ups."

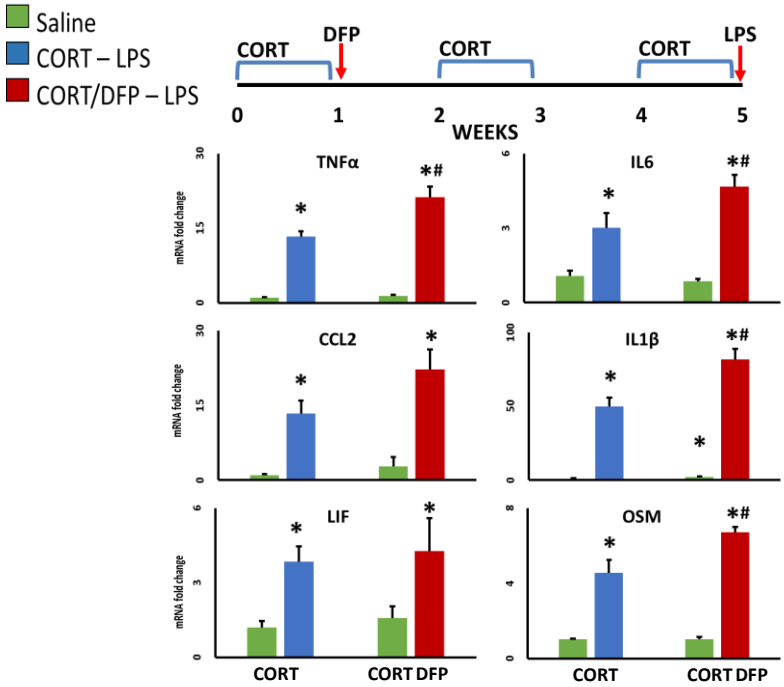

*Figure 2. Mice (n=5) were exposed to CORT+DFP+LPS following the illustrated paradigm. Cytokine mRNA was measured in the cortex 6 hours after LPS exposure. * indicates p<0.05 within treatment (saline vs LPS) # indicates p<0.05 across treatments (CORT vs CORT DFP).*

Following discussion between project PIs regarding mortality rates at the University of Illinois with 8 mg/kg CPO, a dose of 2 mg/kg CPO was used in a similar 5 week GWI paradigm. However, this lower dose of CPO was insufficient to produce measurable changes in cytokine mRNA expression. This study will be repeated with the higher dose of CPO that was used previously, as in the Locker *et al.*, 2017 publication.

An initial dose response study was conducted for dichlorvos (DDVP) from 4-11 mg/kg, i.p. However, exposure to these doses of DDVP failed to produce any significant cholinergic response (i.e. SLUD or seizure activity) or neuroinflammation, as well as no mortality. An amendment adding higher doses (15 and 20 mg/kg, i.p.) has been approved by CDC-NIOSH ACUC and is awaiting approval by ACURO. Once an appropriate dose of DDVP is established, this compound will be tested in both the short- and long-term GWI exposure paradigms utilized for DFP and CPO.

The acetylcholine (ACh) data presented in the previous report has been published in: Miller J.V., LeBouf R.F., Kelly K.A., Michalovicz L.T., Ranpara A., Locker A.R., Miller D.B., O'Callaghan J.P. (2018) The neuroinflammatory phenotype in a mouse model of Gulf War Illness is unrelated to brain regional levels of acetylcholine as measured by quantitative HILIC-UPLC-MS/MS. Tox. Sci. 165(2): 302-313. In these studies, mice were exposed to corticosterone in the drinking water followed by a single exposure to one of the OPs. Brain region-specific responses were found for ACh levels after DFP and CORT+DFP, resulting in amelioration of DFP-induced ACh increase when primed with CORT in the striatum and hippocampus, but not cortex. This was also expanded to CPO and PHY, however, significant amelioration of ACh increase due to CORT priming was not found in our acute mouse model. In comparison to corresponding neuroinflammatory responses, these ACh results indicate an ACh-independent mechanism for GWI-related neuroinflammation.

Using our acute exposure paradigm, where mice receive a week-long exposure to CORT in the drinking water followed by a single injection of DFP or CPO, we had demonstrated previously that the expression of TLR2 was upregulated by CORT+DFP and CORT+CPO exposures compared the OP, AChE inhibitors alone. Additionally, while TLR4 expression levels were unchanged, we found a significant upregulation of the TLR2-regulated, endogenous TLR4 ligands, S100A8 and S100A9. We have evaluated the expression of these genes by qPCR in our 5 week model using DFP (4 mg/kg, i.p.) and CPO (2 mg/kg, i.p.). Interestingly, the long-term exposure paradigm results in not only a significant increase of TLR2, S100A8, and S100A9, but also TLR4 (Figure 1). While there is not a statistically significant difference between CORT+LPS and CORT+DFP/CPO+LPS in this experiment, there is certainly a trend toward a greater effect in the CORT+CPO+LPS group. It is important to note that the fold changes observed for these targets in the 5 week GWI paradigm are much larger than observed previously in the acute exposure (2-10x). Additionally, these changes are observed in the CPO experiment despite the fact that this dose of CPO (2 mg/kg) did not result in significant increases in cytokine expression.

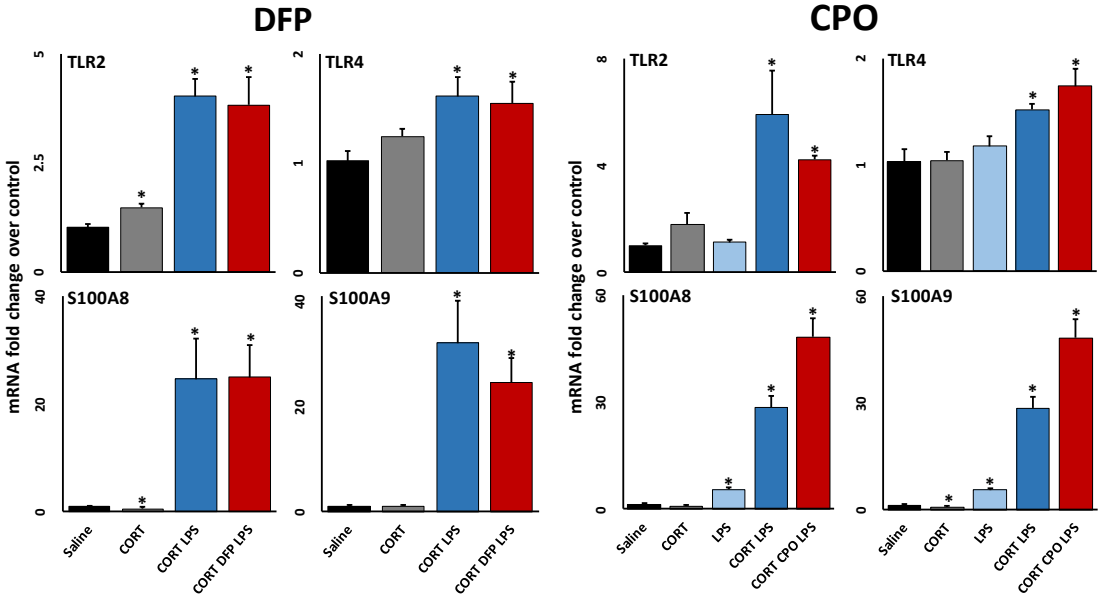

Figure 1. Toll-like receptor signaling is altered in the 5 week GWI paradigm with DFP and CPO. Mice were exposed to CORT in the drinking water for 7 days followed by a single injection of DFP (4 mg/kg, i.p.) or CPO (2 mg/kg, i.p.). Mice were then exposed to CORT every other week for a total of 5 weeks followed by a single injection of LPS (0.5 mg/kg, s.c.). Mice were sacrificed 6 hours after LPS and the cortex was evaluated for the mRNA expression of TLR2, TLR4, S100A8, and S100A9 by qRT-PCR.

The cytokine changes we measure in the brains to assess neuroinflammation likely originate from the brain's resident immune cells, astrocytes and microglia. In previous work for another DoD-funded GWIRP project, histological evaluation of neuroinflammation-associated changes in astrocytes and microglia in our 5 week GWI paradigm with DFP indicated minor morphological changes in these cells that were not observed with the acute exposure paradigm. Thus, we have processed brains from mice exposed to our 5 week GWI paradigm with CPO to investigate if similar changes are seen following exposure to this OP compound. Processing has recently been completed and the histological evaluation is forthcoming for future reporting.

An amendment adding higher doses of DDVP (15 and 20 mg/kg, i.p.) was approved by CDC-NIOSH ACUC and ACURO. In this second dose response study, we evaluated ACh levels along with cytokine expression at 30 minutes and 2 hours post-DDVP, respectively. Interestingly, these animals again experienced no DDVP-induced mortality and only a mild neuroinflammatory response. However, the 20 mg/kg dose demonstrated an increase in ACh (Figure 2) that was similar to what was observed for DFP and CPO (Miller et al., 2018). While this dose of DDVP still did not show a significant change in neuroinflammatory endpoints, this could be due to an issue with the time point chosen (2 hours) or it is possible that DDVP alone is not highly neuroinflammatory. However, previous work has indicated the potential for CORT priming to produce a neuroinflammatory response to a sub-inflammatory dose of an agent. Therefore, we have submitted an amendment to test the 20 mg/kg, i.p. dose of DDVP in the acute GWI paradigm (i.e. 7 days of CORT and 20 mg/kg DDVP on day 8). This amendment has been approved by the CDC-NIOSH IACUC and has been sent to ACURO for further approval. Depending on the results of the acute DDVP study, we will either submit an amendment to use this dose in the 5 week GWI paradigm or perform an additional dosing study with yet higher doses of DDVP.

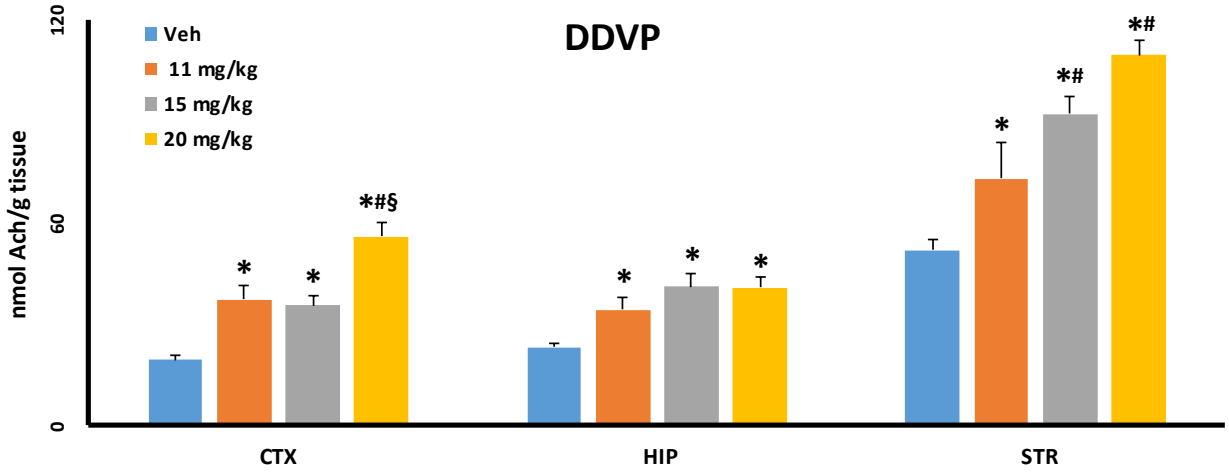

*Figure 2. Acetylcholine levels in discreet brain regions following exposure to DDVP. ACh was measured as described in Miller et al., 2018. Mice were exposed to 11, 15, or 20 mg/kg, i.p. DDVP and sacrificed by microwave fixation 30 minutes following exposure. Brains were dissected and subjected to HILIC-UPLC-MS/MS to measure ACh concentration. Statistical significance measured by one way ANOVA with Fisher LSD post hoc analysis p 0.05 is shown as * vs veh, # vs 11 mg/kg, and § vs 15 mg/kg.*

As a primer to evaluating the expression of plasticity-related genes in our GWI mouse models, we evaluated the expression of *Bdnf, Arc, GluA1, GluA2, NMDAε1*, and *NMDAε2* in two of our existing RNA sequencing datasets: acute CORT+DFP and 5 week GWI. These preliminary data (Figure 3) give promising results for more in-depth analysis of these targets. In particular, the differential results with the NMDA receptor subunits are interesting in that *NMDAε1* showed a DFP-induced response acutely that is maintained in the 5 week GWI dataset and *NMDAε2* responded only in the 5 week dataset.

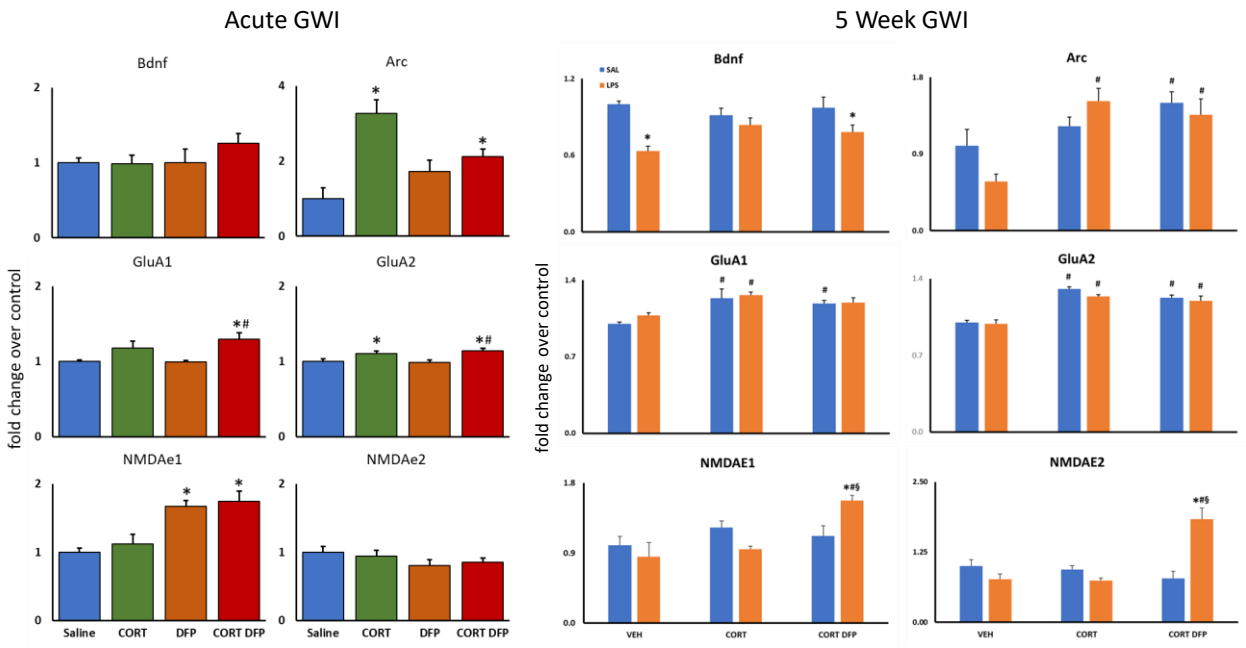

Figure 3. Evaluation of plasticity markers in two GWI RNAseq datasets. The cortex from animals exposed to the acute and 5 week GWI exposure paradigms with DFP was used for genome-wide RNAseq expression analysis. From these existing datasets, the expression of Bdnf, Arc, GluA1, GluA2, NMDAe1, and NMDAe2 were extracted and evaluated for statistical significance.

New Data:

Using our acute exposure paradigm, where mice receive a week-long exposure to CORT in the drinking water followed by a single injection of DFP or CPO, we evaluated two other GW-relevant agents: the pesticide dichlorvos (DDVP; 20 mg/kg, i.p.) and the nerve agent sarin (LD20: 100 µg/kg, s.c.) (Figure 4). Here, we wished to evaluate the neuroinflammatory response to sarin exposure with or without prior CORT to scientifically justify our use of DFP as a sarin surrogate. Similar to our results with DFP and CPO, both DDVP and sarin exposure resulted in significant upregulation of inflammatory cytokines in the brain when combined with prior chronic (7 day) exposure to CORT. These results indicate that our findings with DFP (and all other irreversible AChE inhibitors) are representative of the underlying cellular/molecular effects we believe are the initiating factors for GWI, even in those veterans that may have been exposed to sarin nerve agent in theater. Due to cost restraints, we will not be further pursuing any experiments using sarin; however, we will be evaluating DDVP in our 5 week exposure paradigm.

As per our previous report, a follow-up study was completed using CPO at 8 mg/kg, i.p. in our 5 week exposure paradigm. Unfortunately, we were met with a higher than expected mortality associated with CPO exposure and an unusually high response to the LPS challenge. While these results demonstrate that CORT CPO LPS exposed animals exhibit a significant neuroinflammatory response over controls (Saline or CORT alone), the higher than expected response to LPS challenge resulted in there being no significant difference between CORT LPS and CORT CPO LPS exposed groups (Figure 5). We are continuing to troubleshoot this issue and expect to have additional data to report during the next reporting period.

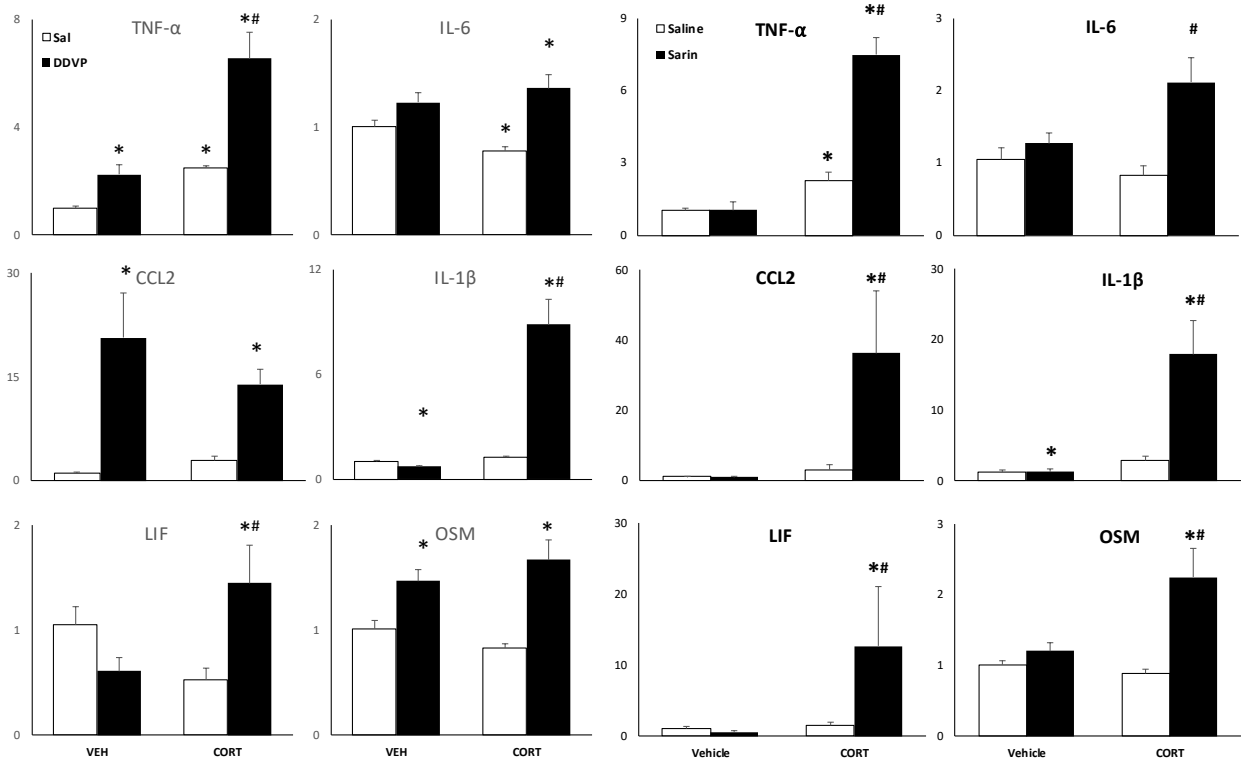

*Figure 4. Prior exposure to corticosterone (CORT) results in a significantly enhanced neuroinflammatory response to dichlorvos (DDVP) and sarin exopsures. Mice were exposed to CORT in the drinking water for 7 days followed by a single injection of DDVP (20 mg/kg, i.p.) or sarin (100 µg/kg, s.c.). Mice were sacrificed 6 hours after DDVP or sarin and the cortex was evaluated for the mRNA expression of TNFα, IL-6, CCL2, IL1β, LIF and OSM by qRT-PCR. * indicates p<0.05 within treatment # indicates p<0.05 across treatments (VEH vs CORT).*

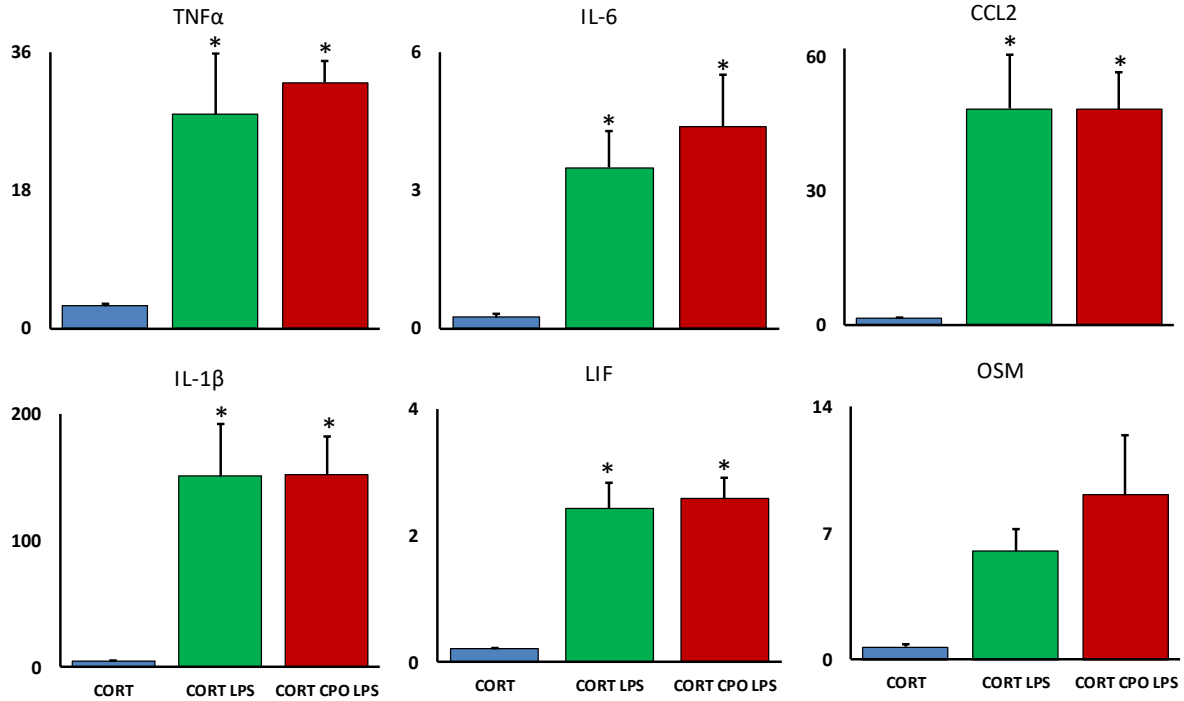

*Figure 5. Exposure to CPO results in significant neuroinflammation in our 5 week paradigm. Mice were exposed to CORT in the drinking water for 7 days followed by a single injection of CPO (8 mg/kg, i.p.). Mice were then exposed to CORT every other week for a total of 5 weeks followed by a single injection of LPS (0.5 mg/kg, s.c.). Mice were sacrificed 6 hours after LPS and the cortex was evaluated for the mRNA expression of inflammatory cytokines. * indicates p<0.05*

In order to continue to expand upon our evaluation of TLR2 signaling in our model of GWI, we evaluated the mRNA expression of TLR2, TLR4, and the endogenous TLR4 ligands: S100A8 and S100A9 by qPCR in our CORT DDVP and CORT Sarin samples, as well as tissue from our CORT PHY studies (Figure 6). While we have demonstrated a significant upregulation of TLR2 mRNA expression using DFP and CPO in a similar paradigm, we do not find much expression change with DDVP or Sarin exposure. However, similar to our previous data, we find a very robust upregulation of the S100A8 and S100A9 mRNAs in both the CORT DDVP and CORT Sarin groups. The S100A8/S100A9 proteins act as alarmins/damage-associated molecular patterns (DAMPs) that trigger the innate immune system to respond cellular stress or damage. Interestingly, S100A8/S100A9 not only bind to TLR4, but also are believed to be produced in response to TLR2 signaling. This suggests that while TLR2 and TLR4 expression may not be universally altered by all iterations of our GWI model, these GW-relevant exposures seem to instigate endogenous DAMP signaling through these receptors. Since this signaling seems to persist/be primed in our 5 week GWI paradigm (see Figure 1), it is possible that persistent DAMP signaling by S100A8/S100A9 could be responsible for the ongoing neuroinflammatory priming we believe is the underlying cause of GWI.

We detected no statistically significant changes in TLR2, TLR4, S100A8, or S100A9 mRNA under any conditions with the reversible AChE inhibitor, PHY. These results support our hypothesis that exposure to the irreversible, organophosphate AChE inhibitors (DFP, CPO, DDVP, and sarin) was the initiating factor in the development of GWI.

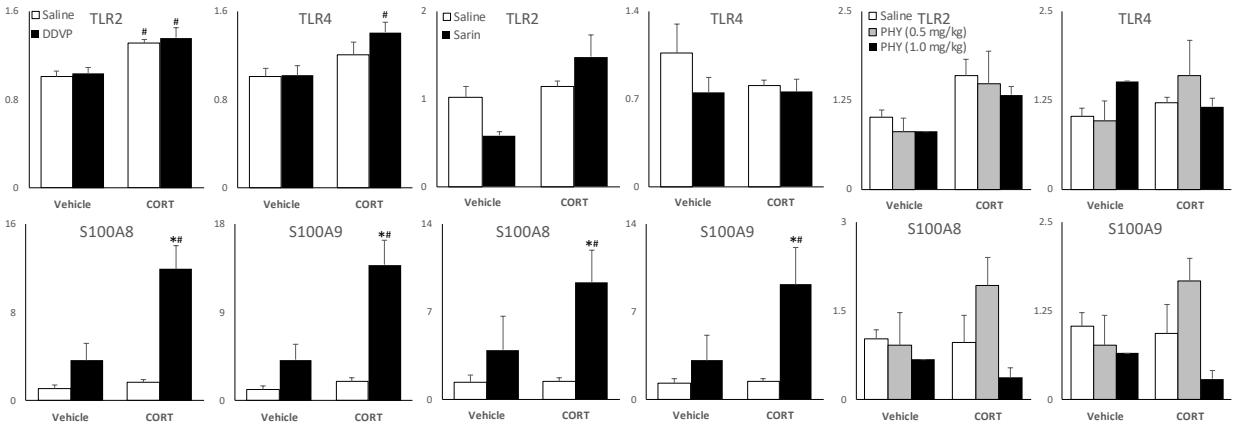

*Figure 6. Toll-like receptor signaling is altered in our acute paradigm using DDVP, Sarin, and PHY. Mice were exposed to CORT in the drinking water for 7 days followed by a single injection of DDVP (20 mg/kg, i.p.), Sarin (100 µg/kg, s.c.) or PHY (0.5 or 1.0 mg/kg, i.p.). Mice were sacrificed 6 hours later and the cortex was evaluated for the mRNA expression of TLR2, TLR4, S100A8, and S100A9 by qRT-PCR. * indicates $p<0.05$ within treatment # indicates $p<0.05$ across treatments (VEH vs CORT).*

To further evaluate the role of TLR2 signaling in our GWI model, we have begun evaluation of a TLR2 inhibitor, ortho-vanillin, as a potential therapeutic for the treatment of GWI. An initial dosing study has been performed and is in the process of being evaluated to determine an effective dose to test in the 5 week GWI paradigm. Considering the discussion above regarding S100A8/S100A9-mediated DAMP signaling, the application of this treatment should help to evaluated if TLR2 signaling is causing the upregulation of S100A8/S100A9 mRNA and thus, signaling through the TLR4 receptor.

The previous animal protocols (GW150051P1, GW150051.03) expired in 10/11-2019 and new protocols were approved 11-2019 by CDC-NIOSH for the work funded through this project. These have been submitted to ACURO and are currently awaiting further approval.

What opportunities for training and professional development has the project provided?

Nothing to Report

How were the results disseminated to communities of interest?

The data accumulated for the acetylcholine study were published in:

Miller J.V., LeBouf R.F., Kelly K.A., Michalovicz L.T., Ranpara A., Locker A.R., Miller D.B., O'Callaghan J.P. (2018) The neuroinflammatory phenotype in a mouse model of Gulf War Illness is unrelated to brain regional levels of acetylcholine as measured by quantitative HILIC-UPLC-MS/MS. Tox. Sci. 165(2): 302-313.

The data from the above publication were presented as part of posters at the Annual Society of Toxicology and Society for Neuroscience meetings:

Miller J.V., Kelly, K.A., Michalovicz L.T., Mouch J.A., Prince N, Boyd J.W., O'Callaghan J.P., Miller D.B. (2018) AChE-independent phosphoprotein signaling in an acute mouse model of Gulf War Illness. 57th Annual Meeting of the Society of Toxicology.

Miller J.V., Kelly K.A., Michalovicz L.T., Mouch J., Prince N., Boy J.W., Miller D.B., O'Callaghan J.P. (2018) Spatiotemporal ACh accumulation and phosphoprotein signaling in a mouse model of Gulf War Illness. 48th Annual Meeting of the Society for Neuroscience.

What do you plan to do during the next reporting period to accomplish the goals?

During the next reporting period, the long-term CPO experiment will be repeated to evaluate neuroinflammation and TLR signaling. 5 week DDVP and PHY experiments will be completed. After the evaluation of the ortho-vanillin (TLR2 inhibitor) dose response study data, we will complete a treatment study with this compound in the 5 week DFP paradigm. We will also continue to evaluate the histological samples from the 5 week GWI paradigm with CPO.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

We have demonstrated and disseminated the acute impacts of organophosphates like DFP and CPO in our acute GWI model. Our recent progress in our 5 week GWI paradigm shows that the acute changes to neuroinflammation and TLR signaling not only persist, but also are added to (i.e. changes in TLR4 in the 5 week GWI but not the acute paradigms) and expanded. The new data gathered from the DDVP and sarin studies supports our hypothesis that irreversible AChE inhibitors are largely involved in the underlying neuroinflammation associated with GWI and further extend the role of TLR signaling in GWI. Furthermore, the Miller et al., 2018 publication presents a novel way to analyze ACh and other small molecule neurotransmitters in brain tissue.

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Organophosphate chemicals like chlorpyrifos and dichlorvos are still readily used as pesticides. Our data indicating that CPO and DDVP exposure, particularly under conditions of high levels of stress hormone, results in significant neuroinflammation helps to identify potential neurological hazards to individuals coming into contact with these chemicals.

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

Significant changes in use or care of human subjects

N/A

Significant changes in use of biohazards and/or select agents

Nothing to report

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

Journal publications.

Miller J.V., LeBouf R.F., Kelly K.A., Michalovicz L.T., Ranpara A., Locker A.R., Miller D.B., O'Callaghan J.P. (2018) The neuroinflammatory phenotype in a mouse model of Gulf War Illness is unrelated to brain regional levels of acetylcholine as measured by quantitative HILIC-UPLC-MS/MS. Tox. Sci. 165(2): 302-313. Locker A.R., Michalovicz L.T., Kelly K.A., Miller J.V., Miller D.B., O'Callaghan J.P. (2017) Corticosterone primes the neuroinflammatory response to Gulf War-relevant organophosphates independently of acetylcholinesterase inhibition. J. Neurochem. 142(3): 444-455

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers, and presentations.

Kelly K.A., Michalovicz L.T., Fornal C., Miller D.B., O'Callaghan J.P., Lasley S.M. (2019) Behavioral and histological evidence of a neuroimmune basis for Gulf War Illness. 58th Annual Meeting of the Society of Toxicology. Miller J.V., Kelly, K.A., Michalovicz L.T., Mouch J.A., Prince N, Boyd J.W., O'Callaghan J.P., Miller D.B. (2018) AChE-independent phosphoprotein signaling in an acute mouse model of Gulf War Illness. 57th Annual Meeting of the Society of Toxicology. Miller J.V., Kelly K.A., Michalovicz L.T., Mouch J., Prince N., Boyd J.W., Miller D.B., O'Callaghan J.P. (2018) Spatiotemporal ACh accumulation and phosphoprotein signaling in a mouse model of Gulf War Illness. 48th Annual Meeting of the Society for Neuroscience.

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

Nothing to report

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: James P. O'Callaghan, Ph.D.
Project Role: Co-PI
Researcher Identifier: orcid.org/0000-0001-8497-4598
Nearest person month worked: 2
Contribution to Project: Organizing and designing experiments, writing/editing manuscripts and abstracts
Funding Support: NIOSH Intramural funds

Name: Kimberly A. Kelly, Ph.D.
Project Role: Post-doctoral Fellow
Nearest person month worked: 2
Contribution to Project: Organizing, designing and executing experiments, data curation and statistical analysis, writing/editing manuscripts and abstracts
Funding Support: NIOSH Intramural funds

Name: Lindsay T. Michalovicz, Ph.D.
Project Role: Post-doctoral Fellow
Nearest person month worked: 2
Contribution to Project: Organizing, designing and executing experiments, data curation and statistical analysis, writing/editing manuscripts and abstracts
Funding Support: NIOSH Intramural funds

Name: Brenda Billig
Project Role: Research Technician
Nearest person month worked: 2
Contribution to Project: Animal tissue collection, data curation
Funding Support: NIOSH Intramural funds

Name: Christopher Felton
Project Role: Research Technician
Nearest person month worked: 2
Contribution to Project: Animal dosing, data curation
Funding Support: NIOSH Intramural funds

Name: Ali Yilmaz
Project Role: Research Technician
Nearest person month worked: 2
Contribution to Project: qPCR for mRNA expression, data curation
Funding Support: NIOSH Intramural funds

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Yes, Dr. Michalovicz has been promoted to an FTE fellow position at NIOSH and is now supported by NIOSH Intramural funds.

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: A collaborative award has been made to Dr. Lasley for this Research Expansion Award and an independent annual report will be forthcoming from his research group.

9. APPENDICES: a copy of Miller et al., 2018 and Locker et al., 2017 is attached.

TOXICOLOGICAL SCIENCES, 165(2), 2018, 302–313

doi: 10.1093/toxsci/kfy130
Advance Access Publication Date: May 28, 2018
Research Article

The Neuroinflammatory Phenotype in a Mouse Model of Gulf War Illness is Unrelated to Brain Regional Levels of Acetylcholine as Measured by Quantitative HILIC-UPLC-MS/MS

Julie V. Miller,* Ryan F. LeBouf,† Kimberly A. Kelly,* Lindsay T. Michalovicz,* Anand Ranpara,† Alicia R. Locker,* Diane B. Miller,* and James P. O'Callaghan*,[1]

*Health Effects Laboratory Division and †Respiratory Health Division, Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, Morgantown, West Virginia 26505

[1]To whom correspondence should be addressed at Health Effects Laboratory Division, Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, 1095 Willowdale Road, Mailstop L-3014, Morgantown, WV 26505. Fax: (304) 285-6220. E-mail: jdo5@cdc.gov.

ABSTRACT

Many veterans of the 1991 Persian Gulf War (GW) returned with a chronic multisymptom illness that has been termed Gulf War Illness (GWI). Previous GWI studies have suggested that exposure to acetylcholinesterase inhibitors (AChEIs) in theater, such as sarin and/or pesticides, may have contributed to the symptomatology of GWI. Additionally, concomitant high physiological stress experienced during the war may have contributed to the initiation of the GWI phenotype. Although inhibition of AChE leading to accumulation of acetylcholine (ACh) will activate the cholinergic anti-inflammatory pathway, the signature symptomatology of GWI has been shown to be associated with neuroinflammation. To investigate the relationship between ACh and neuroinflammation in discrete brain regions, we used our previously established mouse model of GWI, which combines an exposure to a high physiological stress mimic, corticosterone (CORT), with GW-relevant AChEIs. The AChEIs used in this study were diisopropyl fluorophosphate (DFP), chlorpyrifos oxon (CPO), and physostigmine (PHY). After AChEI exposure, ACh concentrations for cortex (CTX), hippocampus (HIP), and striatum (STR) were determined using hydrophilic interaction liquid chromatography with ultraperformance liquid chromatography-tandem-mass spectrometry (MS/MS). CORT pretreatment ameliorated the DFP-induced ACh increase in HIP and STR, but not CTX. CORT pretreatment did not significantly alter ACh levels for CPO and PHY. Further analysis of STR neuroinflammatory biomarkers revealed an exacerbated CORT + AChEI response, which does not correspond to measured brain ACh. By utilizing this new analytical method for discrete brain region analysis of ACh, this work suggests the exacerbated neuroinflammatory effects in our mouse model of GWI are not driven by the accumulation of brain region-specific ACh.

Key words: chlorpyrifos; diisopropyl fluorophosphate; mass spectrometry; neuroinflammation; physostigmine; Gulf War Illness.

Gulf War Illness (GWI) can be described as a persistent and exaggerated form of sickness behavior, of which approximately 30% of veterans of the 1991 Persian Gulf War (GW) suffer from to this day (Smith *et al.*, 2013; Steele, 2000). Symptoms of GWI include fatigue, depression, sleep disruption, muscle and joint pain, gastrointestinal and dermatological maladies, and cognitive impairment (Dursa *et al.*, 2016; Fukuda *et al.*, 1998; Steele, 2000; White *et al.*, 2016). The precise cause(s) of GWI is

unknown, but it has been suggested that in theater exposures to acetylcholinesterase inhibitors (AChEIs), such as the nerve agent sarin, pesticides (eg, chlorpyrifos [CPF] and dichlorvos), and oral nerve agent prophylactics, such as pyridostigmine bromide (PB), may have contributed to the initiation of the illness (Locker *et al.*, 2017; O'Callaghan *et al.*, 2015; RAC, 2014). Additionally, concomitant high physiological stress in theater may have contributed to the etiology of GWI (Koo *et al.*, 2018; Locker *et al.*, 2017; O'Callaghan *et al.*, 2015).

The similarity between the symptomatology of GWI and sickness behavior is suggestive of an underlying neuroinflammatory condition (Dantzer *et al.*, 2008; Godbout *et al.*, 2005) and also is known to be associated with animal models of GWI (Koo *et al.*, 2018; Locker *et al.*, 2017; O'Callaghan *et al.*, 2015). Despite the relationship of neuroinflammation to GWI, and the suggestion that AChEIs play a role in GWI etiology, the underlying cause(s) of GWI have yet to be determined. Several animal studies have shown that irreversible AChEIs, such as soman, sarin, dichlorvos, and diisopropyl fluorophosphate (DFP), as expected, cause a dramatic increase in brain acetylcholine (ACh) levels, specifically in the striatum (STR), hippocampus (HIP), and cortex (CTX) shortly after exposure (Flynn and Wecker, 1986; Russell *et al.*, 1981; Stavinoha *et al.*, 1976). Similar results have been reported for reversible AChEIs, such as physostigmine (PHY) (Hallak and Giacobini, 1986). Exposures to soman (Johnson and Kan, 2010), sarin (Henderson *et al.*, 2002), the sarin surrogate, DFP (Koo *et al.*, 2018; Locker *et al.*, 2017; O'Callaghan *et al.*, 2015), and CPF (Locker *et al.*, 2017) also have been shown to increase proinflammatory mediators in brain hours after AChEI exposure, suggesting a potential role of AChEIs in acute (6–48-h post-AChEI exposure) neuroinflammation. In animal models of GWI that incorporate chronic corticosterone (CORT) exposure prior to AChEI exposures to mimic concomitant high physiological stress likely experienced by service members in theater, a markedly exacerbated neuroinflammatory response occurs for CORT plus irreversible AChEIs when compared with irreversible AChEIs alone (Koo *et al.*, 2018; Locker *et al.*, 2017; O'Callaghan *et al.*, 2015). In contrast, inhibition of brain AChE activity by irreversible AChEIs appears to be blunted in whole brain by prior CORT exposure (CORT priming), suggesting an AChE-independent mechanism for AChEI-related neuroinflammation (Locker *et al.*, 2017).

Although our previous study indicated a CORT-effect on whole brain AChE activity (Locker *et al.*, 2017), it is unclear how this may affect brain region-specific ACh accumulation. Several studies have indicated the potential for glucocorticoid exposure to affect AChE enzyme expression levels in muscle and liver (Askanas *et al.*, 1992; Brank *et al.*, 1998; Weber *et al.*, 1999). Interestingly, Wuppen *et al.* (2010) found that combined exposure to CORT and PHY increased AChE expression in the brain. Thus, measurement of ACh in discrete brain regions following exposure to our model of GWI may offer additional insight as to the role of ACh accumulation in GWI.

In order to elucidate the potential relationship between brain ACh and neuroinflammation, or lack thereof, in GWI etiology, this study combined focused microwave irradiation (O'Callaghan and Sriram, 2004) and hydrophilic interaction liquid chromatography (HILIC)- mass spectrometry (MS/MS) to measure ACh concentrations in discrete brain regions of a mouse model of GWI. Here, in concurrence with our previous study (Locker *et al.*, 2017), several AChEIs were employed, including: DFP, an irreversible AChEI and sarin surrogate; CPF oxon (CPO), the active metabolite of CPF, an irreversible AChEI insecticide used in theater; and PHY, a reversible AChEI that has the ability to cross the blood brain barrier and is similar to PB (Grauer *et al.*, 2000), which was used as a prophylactic against nerve agents in theater. ACh concentrations were elevated after AChEI exposure alone, but CORT + DFP exposure ameliorated AChEI-induced ACh increase in STR and HIP. There was no mixture ameliorating effect on ACh for CPO or PHY. As changes in ACh were most pronounced in the STR, we investigated the neuroinflammatory response to these conditions in this brain area; CORT + DFP and CORT + CPO resulted in exacerbated neuroinflammatory responses when compared with AChEI alone, whereas CORT + PHY exhibited minimal effects. These results suggest an AChE-independent action of DFP and CPO when primed with CORT that is driving the exacerbated neuroinflammatory response in this mouse model of GWI.

MATERIALS AND METHODS

Materials

DFP, PHY, and ethanol (EtOH) were purchased from Sigma-Aldrich Co. (St Louis, Missouri), CPO was purchased from Chem Service, Inc. (West Chester, Pennsylvania), and CORT was purchased from Steraloids Inc. (Newport, Rhode Island). Ultraperformance liquid chromatography-mass spectrometry (UPLC-MS/MS) standards, ACh chloride and acetylcholine-1,1,2,2-d_4 chloride (ACh-d_4) was purchased from Sigma-Aldrich Co. and CDN Isotopes Inc (Pointe-Claire, Quebec, Canada), respectively. HPLC grade reagents were used for all UPLC-MS analyses.

Animals

Adult male C57Bl/6J mice ($n = 5$ or 7/group; 8–12 weeks of age) were purchased from Jackson Laboratory (Bar Harbor, Maine). All mouse procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee of the Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health and U.S. Army Medical Research and Material Command Animal Care and Use Review Office. The animal care program was accredited by AAALAC, International. Upon arrival, mice were individually housed in a temperature- (21°C ± 1°C) and humidity-controlled (50% ± 10%) colony room maintained under filtered positive-pressure ventilation on a 12-h light (06:00 EDT)/12-h dark cycle (18:00 EDT). Mice were individually housed in 12.1 × 5 × 5 in (30.7 × 12.7 × 12.7 cm) plastic cages with hardwood bedding (Teklad sani-chips 7090, Envigo, Indianapolis, Indiana) at a depth of 1–2 cm. Throughout the study, mice were singly housed and given ad libitum access to food (Harlan 7913 irradiated NIH-31 modified 6% rodent chow) and water.

Dosing

Mice ($n = 5$) were exposed to CORT and AChEIs as previously described (Locker *et al.*, 2017). Briefly, intraperitoneal (i.p.) injections of saline (0.9%), peanut oil (CPO vehicle), CPO (8 mg/kg,) DFP (4 mg/kg), or PHY (0.5 mg/kg) were administered in the morning and returned to their home cage. CORT was given in the drinking water (400 mg/l in 1.2% EtOH) for 4 days prior to AChEI or vehicle exposure. CPO, the active, AChE inhibiting metabolite of CPF, was given in place of CPF to avoid variability related to interanimal differences in the bioactivation of CPF to CPO *in vivo* (Cole *et al.*, 2005; Jiang *et al.*, 2010, 2012). For ACh quantification, mice were sacrificed at 30 min (DFP, CPO, and PHY groups) and 45 min (PHY groups) post-AChEI or vehicle exposure by focused microwave irradiation (Muromachi

Microwave Fixation Applicator, model TMW-4012C, Tokyo, Japan; 6 kW 0.9s) to preserve *in vivo* steady state levels of ACh. For qPCR analyses, mice were sacrificed via decapitation at 6-h postexposure to AChEI or vehicle.

Brain Dissection and Preparation

After sacrifice via decapitation or focused-microwave irradiation, whole brains were immediately removed from the skull and the CTX, HIP, and STR were dissected free-hand with fine curved forceps on a thermoelectric cold plate (Model TCP-2; Aldrich Chemical Co., Milwaukee, Wisconsin). Dissected brain regions were frozen and stored at −80°C until subsequent ACh preparation or total RNA isolation.

ACh Quantification in Brain Tissue

Rationale. Measurement of ACh concentrations in brain tissue for ACh accumulation after AChEI exposure have been successfully determined using a combination of pyrolysis and gas chromatography-mass spectrometry (GC-MS), which involves long sample preparations and labor intensive derivatization of ACh (Hong *et al.*, 2013; Stavinoha *et al.*, 1976). To improve sample preparation time and maintain accuracy, liquid chromatography methods have been suggested, but due to the polar nature of ACh and other neurotransmitters alike, reversed phase chromatography exhibits poor retention of these neurotransmitters and can only be used on derivatized samples (Tufi *et al.*, 2015). Cation-exchange or ion-pair chromatography stationary phases would improve ACh retention but use highly concentrated buffers, resulting in electrospray ionization suppression. To avoid retention and suppression limitations, HILIC coupled with mass spectrometry and multiple reaction monitoring has been the emergent and advantageous LC-MS method for neurotransmitter detection and quantitation in biological samples (Peng *et al.*, 2011; Schebb *et al.*, 2008; Uutela *et al.*, 2005; Zhang *et al.*, 2016). HILIC-MS/MS has several advantages over orthogonal ACh quantitative approaches, such as simple and rapid sample preparation and analysis, especially in complex biological matrices, and the ability to differentiate between ACh and the endogenous ACh isomer (iso-ACh), (3-carboxypropyl)trimethylammonium, which is involved in L-carnitine synthesis (Schebb *et al.*, 2008; Zhang *et al.*, 2016). These factors make HILIC-MS/MS an advantageous method for ACh determinations in a variety of biological samples, such as blood (Zhang *et al.*, 2016), cerebrospinal fluid (Zhang *et al.*, 2011), brain microdialysates (Uutela *et al.*, 2005; Zhang *et al.*, 2007), cell culture (Schebb *et al.*, 2008), liver tissue (Wang *et al.*, 2008), and rodent whole brain homogenate (Peng *et al.*, 2011).

Preparation of stock solutions. ACh and ACh-d4 (internal standard; I.S.) were prepared by dissolving between 10 and 25 mg (accurately weighed) in 1 ml water. Stock solutions of ACh and ACh-d4 were prepared by dilution with acetonitrile (ACN) containing 0.1% formic acid and stored at 4°C for no longer than 2 weeks to ensure stability. Fresh standard dilutions were prepared daily. For calibration curve preparation, ACh stock solution was diluted with ACN containing 10.1 nM I.S. (ACh-d4) and 0.1% formic acid, to a final ACh concentration of 5 μM. The remaining standard solutions were diluted with ACN containing 10 nM I.S. The concentrations used for the calibration curve were: 100, 50, 25, 10, 5, 1, and 0.5 nM ACh containing 10 nM ACh-d4. Standards were filtered using Corning regenerated cellulose (RC) filters, with pore size 0.2 μm and 4 mm diameter (Fisher 09-754-3).

Sample preparation. For quantification of ACh, frozen tissue was weighed, diluted with 500–1000 μl ACN with 0.1% formic acid, and sonicated for 90 s (Kontes micro-ultrasonic cell disruptor, Vineland, New Jersey). Sonication in high percent ACN destroys the cell membrane to release ACh and denature proteins. Samples were then centrifuged and ACh-containing supernatant was stored at −20°C until analyses. On the day of analysis, sample supernatants were diluted 1:50 in ACN containing 0.1% formic acid and 10.2 nM ACh-d4 (for a final I.S. concentration of 10 nM in 1 ml). After vortexing for 10 s, samples were filtered using Corning RC filters, with pore size 0.2-μm and 4-mm diameter (Fisher 09-754-3). Samples with ACh < 0.5 nM samples were diluted 1:10 and reanalyzed.

Ultraperformance liquid chromatography-mass spectrometry. For ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analyses, an Acquity H-class UPLC system (Waters Corp., Milford, Massachusetts) equipped with a cooled autosampler, and a CORTECS UPLC HILIC column (Waters 186007105) with dimensions 2.1 × 75 mm, particle size 1.6 μm, and pore size 90 Å was used. The UPLC was connected to a Xevo TQD triple quadrupole mass spectrometer (Waters Corp). The software used for controlling the UPLC and MS was MassLynx and TargetLynx V4.1. For UPLC separation, the mobile phase used was 100 mM ammonium formate (A) and acetonitrile (B), both containing 0.1% formic acid. All mobile phase solutions were filtered and degassed prior to use. The elution gradient was as follows: 0 min (90% B), 0.75 min (60% B) 1.00 min (60% B), 1.25 min (30% B), 1.90 min (30% B), 2.20 min (90% B), and 2.50 min (90%B). The target retention time for ACh and ACh-d4 was 1.41 min and 1.40 min, respectively. The gradient steps after 2.20 min were used to clean and re-equilibrate the column. Total analysis time was 3 min, which includes column equilibration. The injection volume was 10 μl, the flow rate was 0.5 ml/min, and the autosampler was kept below 6°C. For MS/MS data acquisition, the blanks, standards, and analytes were ionized using electrospray ionization in positive mode. The ionization parameters for the Xevo TQD MS were as follows: capillary voltage 3 kV, source temperature 150°C and desolvation temperature 500°C. The analytes of interest, ACh and ACh-d4 (I.S.), were detected using positive ion mode tandem mass spectrometry in multiple reaction monitoring mode (MRM). The MRM transitions measured for ACh were 146→87, 59 and for ACh-d4 (I.S.) were 150→91, 109. For both ACh and ACh-d4 the first transition was used for quantification due to peak intensity and the second was used for identification. The optimized UPLC and MS conditions for ACh and ACh-d4 are summarized in Figure 1. Quantification was based on peak area of the ACh and ACh-d4 peaks. The calibration curve was prepared from the calculated response of the daily prepared standards using the equation

$$\text{Response} = A_{\text{ACh}} \times \left(\frac{\text{I.S. conc}}{A_{\text{I.S.}}}\right),$$

where A_{ACh} is the peak area of the ACh peak at 1.4 min (146→87), I.S. conc is the concentration of the internal standard (ACh-d4), and $A_{\text{I.S.}}$ is the peak area of the I.S. peak at 1.4 min (150→91). With the calculated ACh standard responses, linear regression using 1/*x* weighting was used as the calibration curve to interpolate the sample ACh. The interassay coefficient of variation (CV) for ACh was <8%, including QC brain samples ran up to a year apart, and intra-assay CV was <3%.

RNA Isolation, cDNA Synthesis, and qPCR

The neuroinflammatory RNA markers were determined for STR as described previously for CTX and HIP (Locker *et al.*, 2017). Briefly, total RNA in the STR was isolated using Trizol reagent

UPLC conditions

System	Acquity H-class UPLC
Column	Cortecs UPLC HILIC 1.6µm, 2.1 mm x 75 mm
Mobile phase (A)	100 mM ammonium formate
Mobile phase (B)	Acetonitrile (ACN)
Gradient	0 min (90% B), 0.75 min (60% B) 1.00 min (60% B), 1.25 min (30% B), 1.90 min (30% B), 2.20 min (90% B), and 2.50 min (90%B)
Column temperature	45°C
Sample temperature	6°C
Flow rate	0.5 mL/min
Injection volume	10 µL
Retention time	1.4 min (ACh), 1.4 min (ACh-d4)

MS conditions

Mass spectrometer	Xevo TQD
Ionization mode	+ESI
Capillary voltage	3 kV
Desolvation temperature	500°C
Desolvation gas flow	900 L/h

Optimized MRM

Compound*	MRM transition	Cone voltage (V)	Collision energy (eV)
ACh	**146→87**	25	14
ACh	146→59	25	12
ACh-d4	**150→91**	28	12
ACh-d4	150→109	28	6

**For ACh and ACh-d4, the first transition (bolded) was used for quantification due to peak intensity and the second was used for identification. Quantification was based on peak area of the ACh and ACh-d4 peaks.*

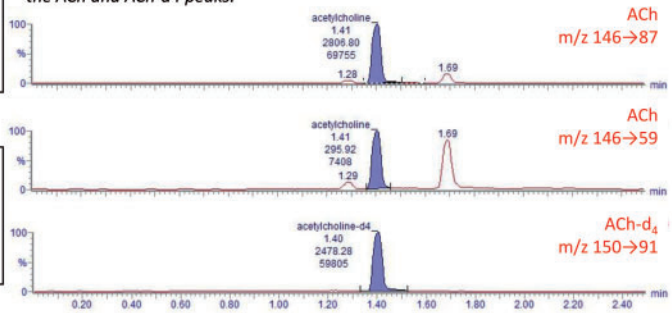

Figure 1. HILIC-UPLC-MS/MS conditions for ACh analysis. The UPLC and MS conditions used for this method are listed in their respective tables, as well as the optimized MRM transitions for ACh (analyte of interest) and ACh-d_4 (internal standard for ACh). Representative MRM chromatograms for brain ACh analysis including peak areas for ACh quantification and identification transitions as well as the quantitation peak for the deuterated internal standard ACh-d_4.

(Thermo Fisher Scientific, Waltham, Massachusetts) and Phase-lock heavy gel (Eppendorf, AG Hamburg, Germany), and purified with RNeasy mini-spin columns (Qiagen, Valencia, California). STR total RNA was reverse transcribed to cDNA using Superscript III and oligo (dT)12–18 primers (Thermo Fisher Scientific). Real-time PCR of glyceraldehyde-3-phosphate dehydrogenase (GAPDH, endogenous control), tumor necrosis factor-alpha (TNFα), interleukin 6 (IL-6), C–C chemokine ligand 2 (CCL2), interleukin 1beta (IL-1β), leukemia inhibitor factor (LIF), and oncostatin M (OSM) was performed using an Applied Biosystems 7500 real-time PCR system (Thermo Fisher Scientific) in combination with TaqMan chemistry. For PCR amplification, 40 cycles were performed in 50 µl (total volume), of which contained 1 µl cDNA, 2.5 µl of primer/probe mix, and 25 µl of Taqman Universal master mix (Thermo Fisher Scientific). Results from our sequence detection software (version 1.7; Applied Biosystems/Thermo Fisher Scientific) were exported into Excel for relative gene expression analysis. Relative gene expression was determined using the comparative threshold (ΔΔCT) method. Fold changes in mRNA expression were calculated after normalization to GAPDH. The resulting normalized ratio values are expressed as fold changes over corresponding controls.

Statistical Analysis

ACh calibration curves, as described in UPLC-MS/MS methods, were constructed and samples interpolated in GraphPad Prism V5 (San Diego, California). Fold change for mRNA using the ΔΔCT method was calculated in Excel and values were exported to GraphPad Prism V5 for further analysis and graph preparation. Bivariate Pearson correlation analysis between ACh concentrations and -ΔΔCT (not log transformed) mRNA in STR was performed using SAS JMP V13 (Cary, North Carolina). Statistical significance for ACh and mRNA expression was determined using 2-way analysis of variance (ANOVA) with Bonferroni post-test. The 2-way ANOVAs conducted on mRNA were performed on log transformed mRNA data. For all statistical analyses, a difference of $p < .05$ was considered statistically significant. ACh is reported as nmol/g tissue mean ± SEM. For mRNA fold change data, error bars reflect SEM.

RESULTS

Focused Microwave Irradiation and HILIC-UPLC-MS/MS Provide a Simple and Rapid Method for ACh Quantitation

The HILIC-UPLC-MS/MS method developed for this study offers a simple, reproducible, and rapid means of quantifying ACh in a complex matrix (brain homogenate). By using focused microwave irradiation as the method of euthanasia, inactivation of endogenous enzymes occurs almost (<0.9 s) simultaneously, as shown previously (Ikarashi *et al.*, 1984; Kobayashi *et al.*, 1980; Stavinoha *et al.*, 1973), therefore addition of AChE inhibitors or similar additives, postsacrifice during sample preparation is unnecessary. Additionally, rapid sample preparation via high percentage acetonitrile and ultrasonic homogenization makes this method advantageous over other ACh methods. Total analysis times for HILIC-UPLC-MS/MS were 3 min per sample, making this method high-throughput. Elution of ACh occurred at 1.41 min and was effectively separated from iso-ACh, which eluted at 1.69 min (Figure 1). The percent coefficient of variation reported in this study was <3% and 8% for intra- and interassay variation, respectively. The vehicle only control mice ACh values for all experiments ($n = 15$) were: CTX (12.1 ± 2.8 nmol/g tissue), HIP (19.1 ± 4.1 nmol/g tissue), and STR (42.5 ± 5.3 nmol/g tissue). These control values are similar to those obtained using focused microwave irradiation and orthogonal ACh quantification methods (Kobayashi *et al.*, 1980).

CORT Priming Ameliorates DFP-Induced Effects on ACh and is Brain Region Specific

The body of literature categorizing GWI as a protracted sickness behavior in conjunction with epidemiology studies suggesting environmental exposures to a host of chemicals, including AChEIs (nerve agents, pesticides, and prophylactics) and concomitant high physiological stress in theater has led to the current rodent model of GWI. Using the exposure paradigm

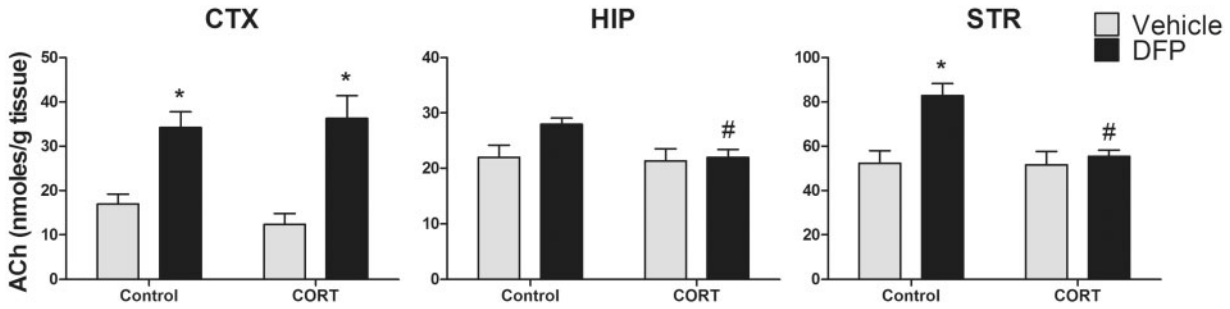

Figure 2. ACh in discrete brain regions after DFP exposure. ACh was measured using our HILIC-UPLC-MS/MS method as described in Materials and Methods. Mice were exposed to control or CORT (400 mg/l in 1.2% EtOH) in the drinking water for 4 days. On the fifth day, mice were exposed to a single i.p. injection of saline or DFP (4 mg/kg) and sacrificed via focused microwave irradiation 30-min postexposure. Whole brain samples were free-hand dissected for CTX, HIP, and STR. Significance was determined using two-way ANOVA with Bonferroni post-test, where * indicates $p < .05$ for vehicle vs DFP or CORT vs CORT+DFP and # indicates $p < .05$ DFP alone vs CORT+DFP. Data are represented as mean ± SEM and $n = 5$.

presented previously (Locker *et al.*, 2017), CORT was administered in the drinking water for 4 days to induce high levels of circulating CORT and on the fifth day, mice were exposed to a single dose of DFP. In this model, we found that DFP significantly reduced AChE activity in whole brain lysates 30 min after exposure, an observation that was significantly ameliorated by prior CORT exposure (Locker *et al.*, 2017). For the current study, at 30-min post-DFP exposure, mice were sacrificed via focused microwave irradiation and discrete brain regions were dissected for ACh analyses. The 30-min time point was selected from previous time course data (30 min, 2-, and 24-h post-DFP), where 30 min showed the maximum ACh concentration. After the 30-min time point, ACh steadily decreased at 2 h (16% reduction from 30-min post-DFP ACh) and 24 h (40% reduction from 30-min post-DFP ACh). Using HILIC-UPLC-MS/MS, ACh concentrations in CTX, HIP, and STR were determined (Figure 2). For HIP and STR, CORT + DFP ACh responses were significantly reduced ($p < .05$) when compared with DFP alone ACh concentrations. However, in the CTX, CORT had no effect on DFP-induced increase in ACh.

CORT Priming Does Not Significantly Ameliorate Irreversible AChEI CPO- or Reversible AChEI PHY-Induced Effects on ACh

In our prior evaluation of AChE activity, we found that prior CORT exposure significantly reversed the dramatic reduction in enzyme activity levels induced by CPO exposure (Locker *et al.*, 2017). However, the reversible AChE inhibitor PHY instigated a significant, yet lesser magnitude, reduction in AChE activity that was not responsive to CORT (Locker *et al.*, 2017). Using the same dosing paradigm as described for DFP, the irreversible inhibitor CPO and reversible inhibitor PHY were studied for ACh effects with and without CORT priming (Figure 3). In HIP and STR at 30-min postexposure to CPO, ACh was significantly increased but CORT priming had no ACh amelioration effects. At 30-min postexposure to PHY, there were no significant differences in CTX, HIP, or STR (data not shown). To determine if this was an exposure time-related effect, mice were sacrificed at 45-min postexposure to PHY. At 45-min postexposure to PHY, all brain regions measured for ACh (CTX, HIP, and STR) were significantly increased, but similarly to CPO, CORT priming had no statistically significant effect on AChEI-induced ACh accumulation (Figure 3). The use of 45-min post-PHY exposure to measure brain ACh accumulation is consistent with previous research using microdialysates of mice exposed to PHY, where the ACh accumulation maxima occurs at 45-min postexposure (Mohr *et al.*, 2013).

CORT Priming Exacerbates Neuroinflammatory Response in STR to Irreversible AChEIs DFP and CPO

In keeping with our previous analyses (Koo *et al.*, 2018; Locker *et al.*, 2017; O'Callaghan *et al.*, 2015), to determine the contribution of ACh levels to the neuroinflammatory response after irreversible AChEI exposure, a panel of neuroinflammation-related cytokines and chemokines were measured at 6-h post-AChEI exposure. This time point was used due to acute response maxima across most neuroinflammatory markers for the AChEIs used in this study (O'Callaghan *et al.*, 2015). The panel of neuroinflammation-related cytokines and chemokines used in this study were TNFα, IL-6, CCL2, IL-1β, LIF, and OSM. After DFP or CPO-alone exposure, the neuroinflammatory panel responses were minimal. For DFP-alone, IL-6 was the only marker measured to have a statistically significant increase in fold change (Figure 4). For CPO-alone, IL-6 was significantly decreased and OSM was significantly increased at 6-h post-exposure (Figure 5). However, for CORT + DFP and CORT + CPO, all neuroinflammatory responses were significantly exacerbated when compared with AChEI alone (Figs. 4 and 5). The only exception is OSM, where CPO alone and CORT + CPO were significantly increased over their respective controls, but were not significantly different from each other, indicating a lack of CORT priming effect on OSM. Bivariate Pearson correlation analysis for STR ACh and measured STR neuroinflammatory responses shows no significant relationship between ACh and cytokine and chemokine mRNA for DFP and CPO, with the exception of a moderate positive correlation for CPO STR ACh and CCL2 ($r = 0.573$, $p = .0103$) and OSM ($r = 0.533$, $p = .0189$). These trends for cytokine and chemokine responses were similar to those found previously for CTX and HIP (Locker *et al.*, 2017).

Reversible AChEI PHY Has Little Impact on Neuroinflammatory Response in STR Alone or in Combination With CORT

To determine the contribution of ACh levels to the neuroinflammatory response after a reversible AChEI exposure; the same panel of neuroinflammation-related cytokines and chemokines were measured at 6-h post-PHY exposure. For the neuroinflammatory panel chosen in this study, the reversible AChEI PHY had no significant differences from control for PHY-alone groups. Additionally, for CORT + PHY, only TNFα and OSM responses were significantly different from PHY alone, albeit minimally in comparison to the exacerbated responses for CORT + DFP and CORT + CPO; results similar to what was observed previously in CTX and HIP (Locker *et al.*, 2017). Bivariate Pearson correlation analysis also showed no statistically

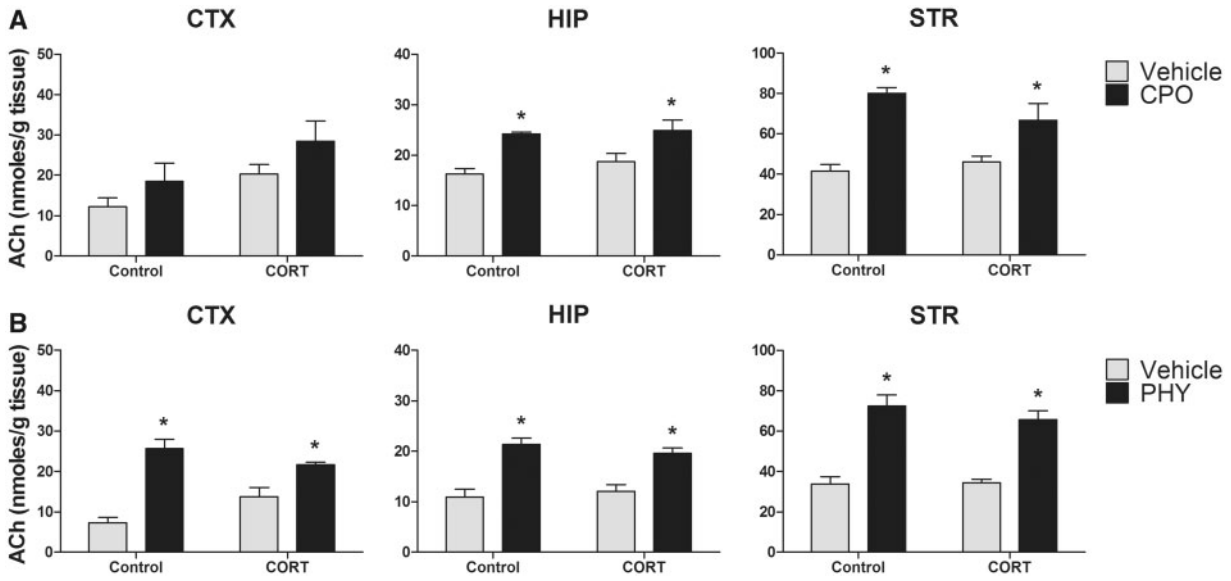

Figure 3. ACh in discrete brain regions after A, irreversible AChEI CPO or B, reversible AChEI PHY exposure. ACh was measured using our HILIC-UPLC-MS/MS method as described in Materials and Methods. Mice were exposed to control or CORT (400 mg/l in 1.2% EtOH) in the drinking water for 4 days. On the fifth day, mice were exposed to a single i.p. injection of peanut oil (for CPO controls), saline (for PHY controls), CPO (8 mg/kg), or PHY (0.5 mg/kg) and sacrificed via focused microwave irradiation 30-min post-CPO and 45-min post-PHY. Whole brain samples were free-hand dissected for CTX, HIP, and STR. Significance was determined using 2-way ANOVA with Bonferroni post-test, where * indicates $p < .05$ for vehicle versus AChEI or CORT versus CORT + AChEI and # indicates $p < .05$ AChEI alone versus CORT + AChEI. Data are represented as mean ± SEM and $n = 5$.

significant correlation between PHY, STR, Ach, and STR mRNA responses for this mouse model of GWI paradigm.

DISCUSSION

Although more than a quarter of a century removed from the GW, a complete understanding of the exposure(s) initiating GWI or viable treatments thereof have yet to be determined (Dursa *et al.*, 2016). It has been suggested that AChEI exposure in theater, such as airborne nerve agents and pesticides, insecticides sprayed on uniforms, and nerve agent prophylactics, were involved in the initiation of GWI (Koo *et al.*, 2018; Locker *et al.*, 2017; O'Callaghan *et al.*, 2015; RAC, 2014; Steele, 2000; White *et al.*, 2016). With these exposures in mind, we included DFP, which is a surrogate for the nerve agent sarin, CPO, which is the oxon and active metabolite of CPF, and PHY, which is a surrogate for PB that is capable of crossing the blood brain barrier (Grauer *et al.*, 2000; Locker *et al.*, 2017), with or without prior exposure to CORT as a physiological stress mimic for testing our GWI model. Here, we found that while exposure to irreversible AChEIs increase the amount of ACh in the brain, prior exposure to CORT abrogates this increase for DFP, an effect not seen with CPO or PHY (Figs. 2 and 3). Furthermore, as STR showed the strongest ACh response, we measured cytokine mRNA expression in this region to further investigate the relationship between AChE and neuroinflammation. In accordance with our previous findings (Locker *et al.*, 2017; O'Callaghan *et al.*, 2015), CORT pretreatment resulted in a significant neuroinflammatory response to DFP and CPO, but not PHY exposure in STR (Figs. 4–6).

The analysis of discrete brain regions prevents the dilution of potential measured effects in whole brain, thus reducing the chance that important changes after xenobiotic exposure go undetected (Gupta, 2004). The heterogeneity of brain regions is especially important to consider for elucidating mechanisms and toxicological endpoints related to AChEI exposure. For example, STR has a higher density of choline acetyltransferase (ChAT), AChE, and ACh concentration than CTX or HIP, as well as the highest rate of ACh accumulation and AChE activity (Gupta, 2004; Stavinoha *et al.*, 1976; Won *et al.*, 2001). ChAT and AChE enzymes have exceedingly fast kinetics and rapid ACh turnover (Bertrand *et al.*, 1994; Jenden *et al.*, 1974; Kobayashi *et al.*, 1980; Stavinoha *et al.*, 1973; Wilson and Harrison, 1961), thus the time needed to dissect after sacrifice, even if it is mere minutes, can significantly alter the resulting analyses. We have shown previously that phosphorylation state, which also has exceedingly fast kinetics (ie, phosphatase and protease enzymes), can be rapidly altered postsacrifice if using live decapitation or other forms of euthanasia that are not focused-microwave irradiation (O'Callaghan and Sriram, 2004). These sample degradation limitations can be overcome by using focused microwave irradiation as the mode of rodent sacrifice, enzyme inactivation and sacrifice occur almost simultaneously (<0.9 s), thereby preserving *in vivo* ACh (Ikarashi *et al.*, 1984; Kobayashi *et al.*, 1980; Stavinoha *et al.*, 1973). In one study, the ACh concentration in whole mouse brain doubled by utilization of focused microwave irradiation instead of euthanasia via decapitation and immediate homogenization alone (Stavinoha *et al.*, 1973). Therefore, focused microwave irradiation for euthanasia allows for the time necessary for discrete brain region dissection without rapid degeneration of the sample. In this study, our control (vehicle only) ACh results for CTX, HIP, and STR were consistent with control mouse ACh levels found in the literature that also used focused microwave irradiation for euthanasia (Kobayashi *et al.*, 1980).

To disentangle the potential association of AChEI-induced accumulation of ACh in discrete brain regions and GWI, we utilized a method that is capable of not only preserving *in vivo* ACh levels postsacrifice, but also rapidly quantifying brain ACh. The HILIC-UPLC-MS/MS method developed for this study offers a simple sample preparation protocol and high-throughput means of quantifying ACh in a complex sample matrix. The

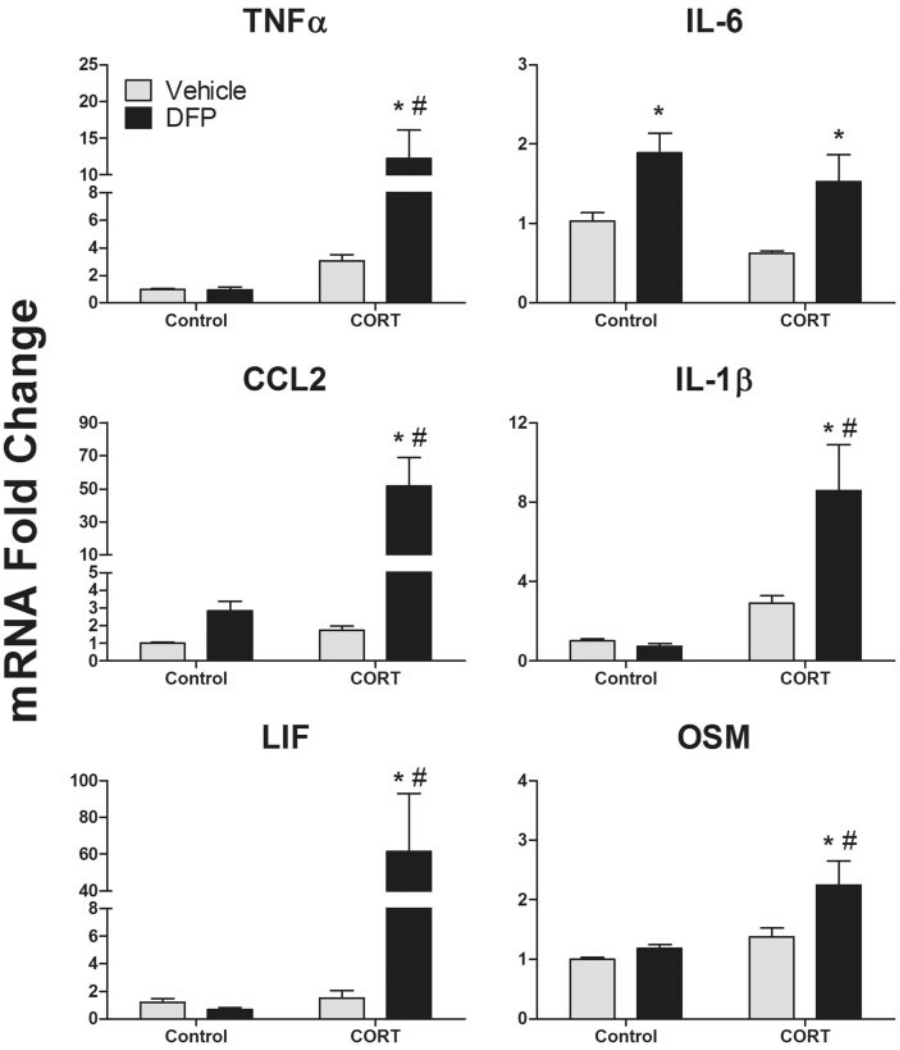

Figure 4. qPCR of measured neuroinflammatory cytokines and chemokines in STR after DFP exposure. Mice were exposed to control or CORT (400 mg/l in 1.2% EtOH) in the drinking water for 4 days. On the fifth day, mice were exposed to a single i.p. injection of saline (DFP vehicle control) or DFP (4 mg/kg) and sacrificed via decapitation 6-h post-DFP. Data were analyzed using the ΔΔCT method. Significance was determined using 2-way ANOVA with Bonferroni post-test where * indicates $p < .05$ for vehicle versus DFP or CORT versus CORT + DFP and # indicates $p < .05$ vehicle versus CORT or DFP alone versus CORT + DFP. Data are expressed as mean fold change ± SEM and $n = 5$.

percent coefficient of variation reported in this study was <3% and 8% for intra- and interassay variation, respectively. These values are consistent with previous studies using HILIC-UPLC-MS/MS to measure ACh in peripheral blood mononuclear cells (Zhang *et al.*, 2016). Additionally, the rapid catalysis of AChE requires a method that is capable of inactivating AChE almost simultaneously at the time of sacrifice to report accurate *in vivo* ACh concentrations. Without this method of euthanasia, the time to sacrifice and dissect can greatly affect the measured ACh during analyses. ACh concentrations observed in this study were consistent with those found in previous DFP exposure studies using orthogonal techniques to measure ACh, such as pyrolysis or ACh derivatization prior to GC-MS, after euthanasia via focused microwave irradiation (Flynn and Wecker, 1986; Russell *et al.*, 1981). Previously, ACh levels in rat STR (150 nmol/g) and HIP (40 nmol/g) 1-h post-DFP exposure have been reported (Flynn and Wecker, 1986). Similarly, in mice, a previous study has reported ACh in CTX (20 nmol/g) and HIP (25 nmol/g) 24-h postexposure to DFP alone. These brain-region specific responses to DFP suggested that it would be advantageous to measure discrete brain regions for ACh accumulation to describe our mouse model of GWI. In our study, at 30-min post-exposure to DFP alone in mice, ACh levels were 34.2 ± 3.6 nmol/g in CTX, 28.0 ± 1.2 nmol/g in HIP, and 82.8 ± 5.5 nmol/g in STR, consistent with those found in the literature (Russell *et al.*, 1981). The development of this rapid and reproducible method allows for ACh levels after AChEI exposure to be quantified and compared for further investigation into the acute response in our GWI model.

Although all 3 AChEIs used in this study are potent AChE inhibitors and used as GW-relevant chemical exposures, it has been shown that not all organophosphate compounds induce toxicity by the same mechanisms (Pope, 1999). Additionally, the potency of AChEIs may not necessarily correlate to acute toxicity *in vivo* (Chambers, 1992). The potential for these AChEIs (DFP, CPO, and PHY) to have divergent mechanisms of toxicity or neuroinflammation and their relevance to in theater exposures makes their inclusion in our mouse model of GWI advantageous. In this study, for our CORT + DFP model, we found an amelioration of AChE inhibition by CORT priming, as evidenced

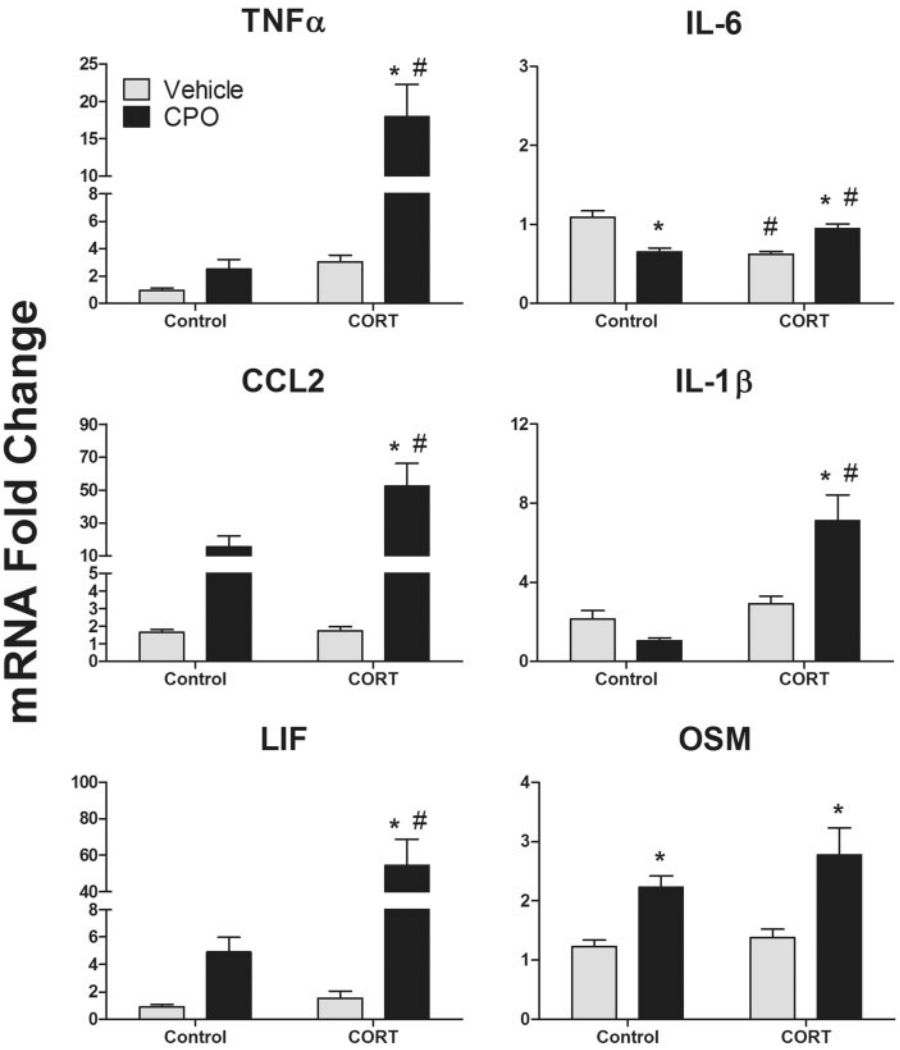

Figure 5. qPCR of measured neuroinflammatory cytokines and chemokines in STR after CPO exposure. Mice were exposed to control or CORT (400 mg/l in 1.2% EtOH) in the drinking water for 4 days. On the fifth day, mice were exposed to a single i.p. injection of peanut oil (CPO vehicle control) or CPO (8 mg/kg) and sacrificed via decapitation 6-h post-CPO. Data were analyzed using the ΔΔCT method. Significance was determined using 2-way ANOVA with Bonferroni post-test where * indicates $p < .05$ for vehicle versus CPO or CORT versus CORT + CPO and # indicates $p < .05$ vehicle versus CORT or CPO alone versus CORT + CPO. Data are expressed as mean fold change ± SEM and $n = 5$.

by a decrease in ACh compared with DFP alone, that is brain region specific. By using this established mouse model of GWI, we found that concomitant stress and DFP exposure (CORT + DFP) portends mixture-driven effects in HIP and STR, whereas CORT priming did not affect AChE inhibition in CTX. Additionally, this unique CORT primed ACh response cannot be extended to all coexposures with GW-relevant AChEIs, whereby CORT + CPO and CORT + PHY did not significantly ameliorate the effects of the AChEIs alone in the HIP and STR. Previously, we have shown at 30 min post-AChEI exposure, AChE specific activity in whole brain is significantly reduced for DFP (10% AChE activity of vehicle control), CPO (25% AChE activity of vehicle control), and PHY (40% AChE activity of vehicle control) alone groups (Locker *et al.*, 2017). However, when primed with CORT, AChE activity is significantly increased over AChEI alone for DFP (50% of vehicle control) and CPO (50% of vehicle control), but not PHY (45% of vehicle control, where PHY alone was 40% of vehicle control) (Locker *et al.*, 2017). These results are similar to our ACh data, where CORT priming dramatically affects and blunts the DFP-induced inhibition of AChE. Additionally, others have shown that swim stress does not affect PHY-induced inhibition of whole brain or CTX cholinesterase (Grauer *et al.*, 2000), supporting our CORT primed PHY ACh results. By dissecting discrete brain regions instead of using whole brain, the regions most affected by CORT priming can be determined and may guide further research on elucidating the potential mechanisms of GWI etiology.

The similarity in symptomatology of sickness behavior and GWI suggests an underlying neuroinflammatory role in GWI etiology (Dantzer and Kelley, 2007; Konsman *et al.*, 2002; Koo *et al.*, 2018; Locker *et al.*, 2017; O'Callaghan *et al.*, 2015; Steele, 2000). The exact mechanism and relationship between identified GW-relevant exposures that may have initiated GWI, such as nerve agent, pesticide, and nerve agent prophylactic AChEIs in addition to concomitant high physiological stress, and neuroinflammation has yet to be elucidated. By quantifying ACh in discrete brain regions, such as STR, HIP, and CTX, the association of ACh levels and relevant neuroinflammatory biomarkers can be determined. Across all AChEIs used in this study, the STR is the most responsive to AChE inhibition; however, ACh levels varied

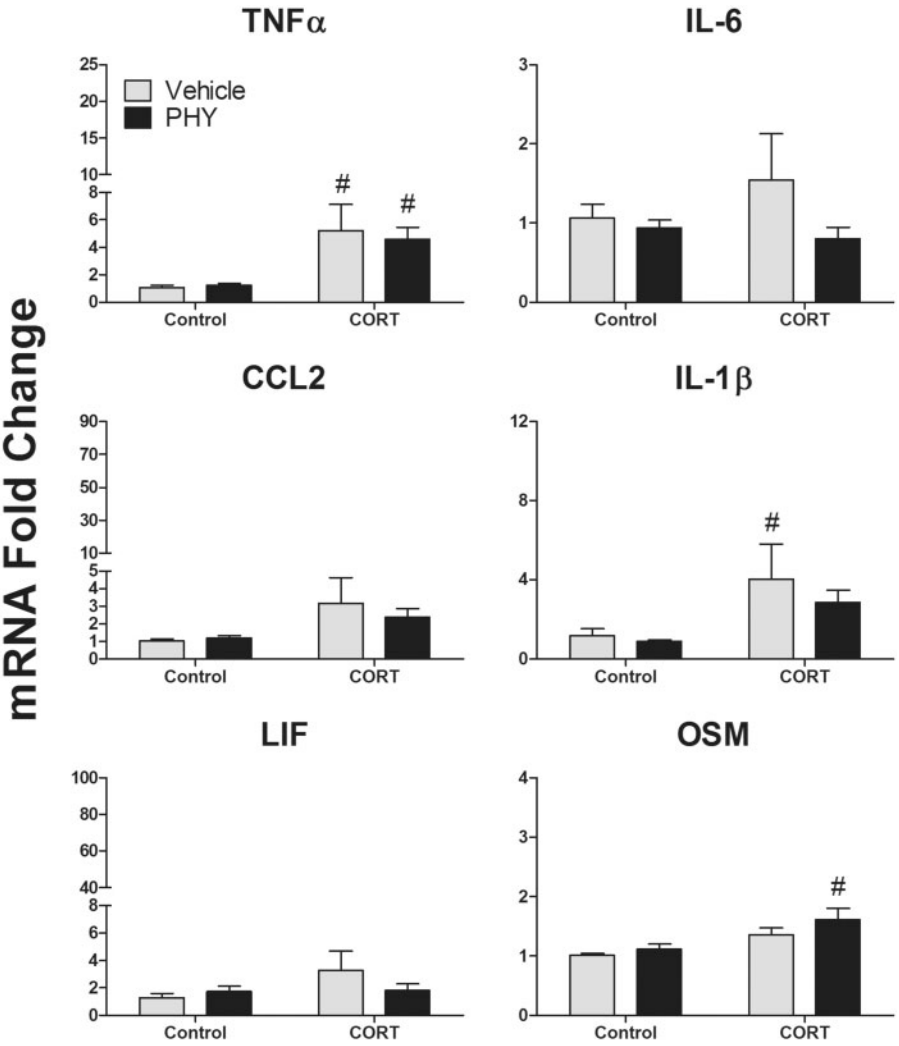

Figure 6. qPCR of measured neuroinflammatory cytokines and chemokines in STR after PHY exposure. Mice were exposed to control or CORT (400 mg/l in 1.2% EtOH) in the drinking water for 4 days. On the fifth day, mice were exposed to a single i.p. injection of saline (PHY vehicle control) or PHY (0.5 mg/kg) and sacrificed via decapitation 6-h post-PHY. Data were analyzed using the ΔΔCT method. Significance was determined using 2-way ANOVA with Bonferroni post-test where * indicates $p < .05$ for vehicle versus PHY or CORT versus CORT + PHY and # indicates $p < .05$ vehicle versus CORT or PHY alone versus CORT + PHY. Data are expressed as mean fold change ± SEM. and $n = 5$.

amongst the AChEIs when primed with CORT. These disparate responses warranted further investigation of the STR and underlying neuroinflammatory signatures. When using the nerve agent sarin surrogate DFP, or CPO, the irreversible AChEIs alone did not significantly affect the neuroinflammatory panel used in this study. However, when DFP or CPO-exposed mice were primed with CORT, almost all of the neuroinflammatory biomarker responses measured were significantly exacerbated (Figs 4 and 5). Although these dramatic neuroinflammatory responses in our GWI mouse model using CORT + DFP and CORT + CPO were significant, there was no significant association between the STR cytokines or chemokines and STR ACh levels, which was verified statistically with bivariate Pearson correlation analysis, with the exception of CPO STR ACh and CPO STR CCL2 and OSM. Although STR CCL2 and ACh were moderately correlated for CPO, it is important to note that CORT + CPO CCL2 is significantly increased (fold change 52.4 ± 13.6) compared with CPO alone (fold change 15.6 ± 6.6), but there is no significant difference between CPO ACh (80.1 ± 3.0 nmol/g) and CORT + CPO ACh (66.7 ± 8.4 nmol/g). This lack of association between STR ACh levels and neuroinflammatory biomarkers used in this study suggest that the exacerbated neuroinflammatory response to CORT primed DFP or CPO exposure in our model of GWI may be driven by AChE-independent mechanisms, such as disruption of critical intracellular signaling pathways related to inflammation. In our previous study, we found a similar exacerbated neuroinflammatory signature in the CTX and HIP for DFP and CPO (Locker *et al.*, 2017; O'Callaghan *et al.*, 2015). Although these results do not indicate a significant link between ACh levels and neuroinflammation in our model of GWI, they do provide evidence for using discrete brain regions instead of whole brain for both ACh and neuroinflammatory biomarkers when evaluating potential etiological mechanisms of GWI.

To determine if a GW-relevant reversible AChEI exhibits the same neuroinflammatory effects as the irreversible AChEIs DFP and CPO, we used PHY, which is considered a surrogate for the nerve agent oral prophylactic, PB, capable of crossing the blood brain barrier (Grauer *et al.*, 2000). PB has been previously identified in epidemiological studies as a potential exposure related

to GWI (Golomb, 2008; Steele *et al.*, 2011). However, it has been shown that PB does not cross the blood brain barrier, even during times of stress, which can increase permeability of the blood brain barrier (Grauer *et al.*, 2000). By using PHY in this study, we can determine the effects of a GW-relevant reversible AChEI. In the neuroinflammatory panel used for this study, PHY alone did not significantly affect the measured cytokine or chemokine responses 6-h postexposure in the STR, whereas ACh levels at 45-min postexposure were significantly increased for PHY and CORT + PHY. This similar neuroinflammatory signature was determined previously for PHY and CORT primed PHY responses in CTX and HIP (Locker *et al.*, 2017). Additionally, there was no significant relationship between ACh levels and the neuroinflammatory biomarkers for any of the brain regions used in this study for PHY. Neuroinflammatory profiles for DFP- and CPO-exposed mice follow a similar pattern, with an exacerbated mixture response with CORT, whereas the reversible AChEI PHY showed little to no effect. From this data, DFP and CPO are more advantageous for a mouse model of GWI than PHY.

In conclusion, we have developed a rapid, simple, and reproducible method for measuring ACh in discrete brain regions following AChEI exposure. By including this method in our mouse model of GWI, brain region-specific effects driving the GWI phenotype can be elucidated. Specifically, we found that amelioration of ACh accumulation via CORT priming is unique to DFP exposed mice in HIP and STR, whereas CORT priming had little to no effect on CPO- or PHY-induced ACh accumulation in the same brain areas. Despite these disparate ACh levels, CORT priming with the irreversible AChEIs DFP and CPO elicited an exacerbated neuroinflammatory response in comparison to AChEI-alone exposure, but PHY did not significantly affect the neuroinflammatory response alone or when combined with CORT. This exacerbated neuroinflammatory response and amelioration of AChEI-induced ACh accumulation to CORT priming and irreversible AChEI exposure further validates this GWI model. In light of these findings, discrete brain region analysis and the inclusion of exogenous CORT to account for concomitant high physiological stress are vital to GWI model development and analyses. Additionally, the disassociation of ACh and neuroinflammatory biomarkers used in this study suggests that GWI may be due to brain region-specific off-target or secondary mechanisms of AChEI exposure and not AChE inhibition. Toxic effects due to non-AChE targets of AChEI exposure have been suggested previously (Bushnell *et al.*, 1993; Pope *et al.*, 1992; Ray and Richards, 2001; van Dongen and Wolthuis, 1989). Organophosphate exposures have been shown to interact with other biomolecular targets via phosphorylation of nucleophilic serine residues (O'Neill, 1981), DNA methylation (Mehl *et al.*, 1994), or disruption of neuropeptide metabolism (Kubek *et al.*, 1997; Richards *et al.*, 2000). Further research investigating GW-relevant exposures and their potential non-AChE targets and corresponding effects must be performed to elucidate the underlying mechanism(s) of GWI etiology.

ACKNOWLEDGMENTS

We appreciate the excellent technical assistance provided by Brenda K. Billig, Christopher M. Felton, and Ali Yilmaz. Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the Department of Defense. The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention. Mention of any company or product does not constitute endorsement by the National Institute for Occupational Safety and Health (NIOSH), Centers for Disease Control and Prevention (CDC).

FUNDING

This work was supported by Congressionally Directed Medical Research Programs: Gulf War Illness Research Program Grants (GW120037, GW120045, GW150051) and intramural funds from the Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health.

REFERENCES

Askanas, V., McFerrin, J., Park-Matsumoto, Y. C., Lee, C. S., and Engel, W. K. (1992). Glucocorticoid increases acetylcholinesterase and organization of the postsynaptic membrane in innervated cultured human muscle. *Exp. Neurol.* **115**, 368–375.

Bertrand, N., Beley, P., and Beley, A. (1994). Brain fixation for acetylcholine measurements. *J. Neurosci. Methods* **53**, 81–85.

Brank, M., Zajc-Kreft, K., Kreft, S., Komel, R., and Grubic, Z. (1998). Biogenesis of acetylcholinesterase is impaired, although its mRNA level remains normal, in the glucocorticoid-treated rat skeletal muscle. *Eur. J. Biochem.* **251**, 374–381.

Bushnell, P. J., Pope, C. N., and Padilla, S. (1993). Behavioral and neurochemical effects of acute chlorpyrifos in rats: Tolerance to prolonged inhibition of cholinesterase. *J. Pharmacol. Exp. Ther.* **266**, 1007–1017.

Chambers, J. E. (1992). The role of target-site activation of phosphorothionates in acute toxicity. In *Organophosphates, Chemistry, Fate and Effects* (J. E. Chambers and P. E. Levi, Eds.), pp. 229–239. Academic Press, San Diego, CA.

Cole, T. B., Walter, B. J., Shih, D. M., Tward, A. D., Lusis, A. J., Timchalk, C., Richter, R. J., Costa, L. G., and Furlong, C. E. (2005). Toxicity of chlorpyrifos and chlorpyrifos oxon in a transgenic mouse model of the human paraoxonase (PON1) Q192R polymorphism. *Pharmacogenet. Genomics* **15**, 589–598.

Dantzer, R., and Kelley, K. W. (2007). Twenty years of research on cytokine-induced sickness behavior. *Brain Behav. Immun.* **21**, 153–160.

Dantzer, R., O'Connor, J. C., Freund, G. G., Johnson, R. W., and Kelley, K. W. (2008). From inflammation to sickness and depression: When the immune system subjugates the brain. *Nat. Rev. Neurosci.* **9**, 46–56.

Dursa, E. K., Barth, S. K., Schneiderman, A. I., and Bossarte, R. M. (2016). Physical and mental health status of Gulf War and Gulf Era veterans: Results from a large population-based epidemiological study. *J. Occup. Environ. Med.* **58**, 41–46.

Flynn, C. J., and Wecker, L. (1986). Elevated choline levels in brain. A non-cholinergic component of organophosphate toxicity. *Biochem. Pharmacol.* **35**, 3115–3121.

Fukuda, K., Nisenbaum, R., Stewart, G., Thompson, W. W., Robin, L., Washko, R. M., Noah, D. L., Barrett, D. H., Randall, B., and Herwaldt, B. L. (1998). Chronic multisymptom illness affecting Air Force veterans of the Gulf War. *Jama* **280**, 981–988.

Godbout, J. P., Chen, J., Abraham, J., Richwine, A. F., Berg, B. M., Kelley, K. W., and Johnson, R. W. (2005). Exaggerated neuroinflammation and sickness behavior in aged mice following activation of the peripheral innate immune system. *Faseb J.* **19**, 1329–1331.

Golomb, B. A. (2008). Acetylcholinesterase inhibitors and Gulf War illnesses. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 4295–4300.

Grauer, E., Alkalai, D., Kapon, J., Cohen, G., and Raveh, L. (2000). Stress does not enable pyridostigmine to inhibit brain cholinesterase after parenteral administration. *Toxicol. Appl. Pharmacol.* **164**, 301–304.

Gupta, R. C. (2004). Brain regional heterogeneity and toxicological mechanisms of organophosphates and carbamates. *Toxicol. Mech. Methods* **14**, 103–1080.

Hallak, M., and Giacobini, E. (1986). Relation of brain regional physostigmine concentration to cholinesterase activity and acetylcholine and choline levels in rat. *Neurochem. Res.* **11**, 1037–1048.

Henderson, R. F., Barr, E. B., Blackwell, W. B., Clark, C. R., Conn, C. A., Kalra, R., March, T. H., Sopori, M. L., Tesfaigzi, Y., and Menache, M. G. (2002). Response of rats to low levels of sarin. *Toxicol. Appl. Pharmacol.* **184**, 67–76.

Hong, J. Y., Park, N. H., Oh, M. S., Lee, H. S., Pyo, H., and Hong, J. (2013). Profiling analysis of biogenic amines and their acidic metabolites in mouse brain tissue using gas chromatography-tandem mass spectrometry. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **940**, 94–103.

Ikarashi, Y., Maruyama, Y., and Stavinoha, W. B. (1984). Study of the use of the microwave magnetic field for the rapid inactivation of brain enzymes. *Jpn. J. Pharmacol.* **35**, 371–387.

Jenden, D. J., Choi, L., Silverman, R. W., Steinborn, J. A., Roch, M., and Booth, R. A. (1974). Acetylcholine turnover estimation in brain by gas chromatography-mass spectrometry. *Life Sci.* **14**, 55–63.

Jiang, W., Duysen, E. G., Hansen, H., Shlyakhtenko, L., Schopfer, L. M., and Lockridge, O. (2010). Mice treated with chlorpyrifos or chlorpyrifos oxon have organophosphorylated tubulin in the brain and disrupted microtubule structures, suggesting a role for tubulin in neurotoxicity associated with exposure to organophosphorus agents. *Toxicol. Sci.* **115**, 183–193.

Jiang, W., Duysen, E. G., and Lockridge, O. (2012). Mice treated with a nontoxic dose of chlorpyrifos oxon have diethoxyphosphotyrosine labeled proteins in blood up to 4 days post exposure, detected by mass spectrometry. *Toxicology* **295**, 15–22.

Johnson, E. A., and Kan, R. K. (2010). The acute phase response and soman-induced status epilepticus: Temporal, regional and cellular changes in rat brain cytokine concentrations. *J. Neuroinflammation* **7**, 40.

Kobayashi, H., Yuyama, A., Matsusaka, N., Takeno, K., and Yanagiya, I. (1980). Effect of methylmercury on brain acetylcholine concentration and turnover in mice. *Toxicol. Appl. Pharmacol.* **54**, 1–8.

Konsman, J. P., Parnet, P., and Dantzer, R. (2002). Cytokine-induced sickness behaviour: Mechanisms and implications. *Trends Neurosci.* **25**, 154–159.

Koo, B. B., Michalovicz, L. T., Calderazzo, S., Kelly, K. A., Sullivan, K., Killiany, R. J., and O'Callaghan, J. P. (2018). Corticosterone potentiates DFP-induced neuroinflammation and affects high-order diffusion imaging in a rat model of Gulf War Illness. *Brain Behav. Immun.* **67**, 42–46.

Kubek, M. J., Shih, T. M., and Meyerhoff, J. L. (1997). Thyrotropin-releasing hormone (TRH) is markedly increased in the rat brain following soman-induced convulsions. *Brain Res.* **747**, 328–331.

Locker, A. R., Michalovicz, L. T., Kelly, K. A., Miller, J. V., Miller, D. B., and O'Callaghan, J. P. (2017). Corticosterone primes the neuroinflammatory response to Gulf War Illness-relevant organophosphates independently of acetylcholinesterase inhibition. *J. Neurochem.* **142**, 444–455.

Mehl, A., Schanke, T. M., Johnsen, B. A., and Fonnum, F. (1994). The effect of trichlorfon and other organophosphates on prenatal brain development in the guinea pig. *Neurochem. Res.* **19**, 569–574.

Mohr, F., Zimmermann, M., and Klein, J. (2013). Mice heterozygous for AChE are more sensitive to AChE inhibitors but do not respond to BuChE inhibition. *Neuropharmacology* **67**, 37–45.

O'Callaghan, J. P., Kelly, K. A., Locker, A. R., Miller, D. B., and Lasley, S. M. (2015). Corticosterone primes the neuroinflammatory response to DFP in mice: Potential animal model of Gulf War Illness. *J. Neurochem.* **133**, 708–721.

O'Callaghan, J. P., and Sriram, K. (2004). Focused microwave irradiation of the brain preserves in vivo protein phosphorylation: Comparison with other methods of sacrifice and analysis of multiple phosphoproteins. *J. Neurosci. Methods* **135**, 159–168.

O'Neill, J. J. (1981). Non-cholinesterase effects of anticholinesterases. *Fundam. Appl. Toxicol.* **1**, 154–160.

Peng, L., Jiang, T., Rong, Z., Liu, T., Wang, H., Shao, B., Ma, J., Yang, L., Kang, L., Shen, Y., *et al.* (2011). Surrogate based accurate quantification of endogenous acetylcholine in murine brain by hydrophilic interaction liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **879**, 3927–3931.

Pope, C. N. (1999). Organophosphorus pesticides: Do they all have the same mechanism of toxicity? *J. Toxicol. Environ. Health B Crit. Rev.* **2**, 161–181.

Pope, C. N., Chakraborti, T. K., Chapman, M. L., and Farrar, J. D. (1992). Long-term neurochemical and behavioral effects induced by acute chlorpyrifos treatment. *Pharmacol. Biochem. Behav.* **42**, 251–256.

RAC. (2014). *Gulf War Illness and the Health of Gulf War Veterans: Research Update and Recommendations, 2009-2013*. US Government Printing Office, Boston, MA.

Ray, D. E., and Richards, P. G. (2001). The potential for toxic effects of chronic, low-dose exposure to organophosphates. *Toxicol. Lett.* **120**, 343–351.

Richards, P. G., Johnson, M. K., and Ray, D. E. (2000). Identification of acylpeptide hydrolase as a sensitive site for reaction with organophosphorus compounds and a potential target for cognitive enhancing drugs. *Mol. Pharmacol.* **58**, 577–583.

Russell, R. W., Carson, V. G., Booth, R. A., and Jenden, D. J. (1981). Mechanisms of tolerance to the anticholinesterase, DFP: Acetylcholine levels and dynamics in the rat brain. *Neuropharmacology* **20**, 1197–1201.

Schebb, N. H., Fischer, D., Hein, E. M., Hayen, H., Krieglstein, J., Klumpp, S., and Karst, U. (2008). Fast sample preparation and liquid chromatography-tandem mass spectrometry method for assaying cell lysate acetylcholine. *J. Chromatogr. A* **1183**, 100–107.

Smith, B. N., Wang, J. M., Vogt, D., Vickers, K., King, D. W., and King, L. A. (2013). Gulf war illness: Symptomatology among veterans 10 years after deployment. *J. Occup. Environ. Med.* **55**, 104–110.

Stavinoha, W. B., Modak, A. T., and Weintraub, S. T. (1976). Rate of accumulation of acetylcholine in discrete regions of the rat brain after dichlorvos treatment. *J. Neurochem.* **27**, 1375–1378.

Stavinoha, W. B., Weintraub, S. T., and Modak, A. T. (1973). The use of microwave heating to inactivate cholinesterase in the rat brain prior to analysis for acetylcholine. *J. Neurochem.* **20**, 361–371.

Steele, L. (2000). Prevalence and patterns of Gulf War illness in Kansas veterans: Association of symptoms with characteristics of person, place, and time of military service. *Am. J. Epidemiol.* **152**, 992–1002.

Steele, L., Sastre, A., Gerkovich, M. M., and Cook, M. R. (2011). Complex factors in the etiology of Gulf War illness: Wartime exposures and risk factors in veteran subgroups. *Environ. Health Perspect.* **120**, 112–118.

Tufi, S., Lamoree, M., de Boer, J., and Leonards, P. (2015). Simultaneous analysis of multiple neurotransmitters by hydrophilic interaction liquid chromatography coupled to tandem mass spectrometry. *J. Chromatogr. A* **1395**, 79–87. 10.1016/j.chroma.2015.03.056.

Uutela, P., Reinila, R., Piepponen, P., Ketola, R. A., and Kostiainen, R. (2005). Analysis of acetylcholine and choline in microdialysis samples by liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **19**, 2950–2956.

van Dongen, C. J., and Wolthuis, O. L. (1989). On the development of behavioral tolerance to organophosphates. I: Behavioral and biochemical aspects. *Pharmacol. Biochem. Behav.* **34**, 473–481.

Wang, Y., Wang, T., Shi, X., Wan, D., Zhang, P., He, X., Gao, P., Yang, S., Gu, J., and Xu, G. (2008). Analysis of acetylcholine, choline and butyrobetaine in human liver tissues by hydrophilic interaction liquid chromatography-tandem mass spectrometry. *J. Pharm. Biomed. Anal.* **47**, 870–875.

Weber, U., Brank, M., and Grubic, Z. (1999). Glucocorticoids differentially control synthesis of acetylcholinesterase and butyrylcholinesterase in rat liver and brain. *Chem. Biol. Interact.* **119–120**, 341–347.

White, R. F., Steele, L., O'Callaghan, J. P., Sullivan, K., Binns, J. H., Golomb, B. A., Bloom, F. E., Bunker, J. A., Crawford, F., Graves, J. C., *et al.* (2016). Recent research on Gulf War illness and other health problems in veterans of the 1991 Gulf War: Effects of toxicant exposures during deployment. *Cortex* **74**, 449–475.

Wilson, I. B., and Harrison, M. A. (1961). Turnover number of acetyl-cholinesterase. *J. Biol. Chem.* **236**, 2292–2295.

Won, Y. K., Liu, J., Olivier, K., Jr., Zheng, Q., and Pope, C. N. (2001). Age-related effects of chlorpyrifos on acetylcholine release in rat brain. *Neurotoxicology* **22**, 39–48.

Wuppen, K., Oesterle, D., Lewicka, S., Kopitz, J., and Plaschke, K. (2010). A subchronic application period of glucocorticoids leads to rat cognitive dysfunction whereas physostigmine induces a mild neuroprotection. *J. Neural Transm. (Vienna)* **117**, 1055–1065.

Zhang, C., Xia, Y., Jiang, W., Wang, C., Han, B., and Hao, J. (2016). Determination of non-neuronal acetylcholine in human peripheral blood mononuclear cells by use of hydrophilic interaction ultra-performance liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **1022**, 265–273.

Zhang, X., Rauch, A., Lee, H., Xiao, H., Rainer, G., and Logothetis, N. K. (2007). Capillary hydrophilic interaction chromatography/mass spectrometry for simultaneous determination of multiple neurotransmitters in primate cerebral cortex. *Rapid Commun. Mass Spectrom.* **21**, 3621–3628. 10.1002/rcm.3251.

Zhang, Y., Tingley, F. D., 3rd, Tseng, E., Tella, M., Yang, X., Groeber, E., Liu, J., Li, W., Schmidt, C. J., and Steenwyk, R. (2011). Development and validation of a sample stabilization strategy and a UPLC-MS/MS method for the simultaneous quantitation of acetylcholine (ACh), histamine (HA), and its metabolites in rat cerebrospinal fluid (CSF). *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **879**, 2023–2033.

Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2017 | 142 | 444–455 doi: 10.1111/jnc.14071

ORIGINAL ARTICLE

Corticosterone primes the neuroinflammatory response to Gulf War Illness-relevant organophosphates independently of acetylcholinesterase inhibition

Alicia R. Locker, Lindsay T. Michalovicz, Kimberly A. Kelly, Julie V. Miller, Diane B. Miller and James P. O'Callaghan

Health Effects Laboratory Division, Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, Morgantown, West Virginia, USA

Abstract

Gulf War Illness (GWI) is a chronic multi-symptom disorder affecting veterans of the 1991 Gulf War. Among the symptoms of GWI are those associated with sickness behavior, observations suggestive of underlying neuroinflammation. We have shown that exposure of mice to the stress hormone, corticosterone (CORT), and to diisopropyl fluorophosphate (DFP), as a nerve agent mimic, results in marked neuroinflammation, findings consistent with a stress/neuroimmune basis of GWI. Here, we examined the contribution of irreversible and reversible acetylcholinesterase (AChE) inhibitors to neuroinflammation in our mouse model of GWI. Male C57BL/6J mice received 4 days of CORT (400 mg/L) in the drinking water followed by a single dose of chlorpyrifos oxon (CPO; 8 mg/kg, i.p.), DFP (4 mg/kg, i.p.), pyridostigmine bromide (PB; 3 mg/kg, i.p.), or physostigmine (PHY; 0.5 mg/kg, i.p.). CPO and DFP alone caused cortical and hippocampal neuroinflammation assessed by qPCR of tumor necrosis factor-alpha, IL-6, C–C chemokine ligand 2, IL-1β, leukemia inhibitory factor and oncostatin M; CORT pretreatment markedly augmented these effects. Additionally, CORT exposure prior to DFP or CPO enhanced activation of the neuroinflammation signal transducer, signal transducer and activator of transcription 3 (STAT3). In contrast, PHY or PB alone or with CORT pretreatment did not produce neuroinflammation or STAT3 activation. While all of the CNS-acting AChE inhibitors (DFP, CPO, and PHY) decreased brain AChE activity, CORT pretreatment abrogated these effects for the irreversible inhibitors. Taken together, these findings suggest that irreversible AChE inhibitor-induced neuroinflammation and particularly its exacerbation by CORT, result from non-cholinergic effects of these compounds, pointing potentially to organophosphorylation of other neuroimmune targets.

Keywords: chlorpyrifos, diisopropyl fluorophosphate, neuroinflammation, physostigmine, pyridostigmine bromide, STAT3.

J. Neurochem. (2017) **142**, 444–455.

Approximately 200 000 soldiers that served in the 1991 Persian Gulf War returned with clinical symptoms that include chronic fatigue, headache, cognitive impairment, depression, muscle and joint pain, and gastrointestinal issues, among others. This multi-symptom illness has been termed Gulf War Illness (GWI) (Steele 2000; Golomb 2008;

Received April 11, 2017; revised manuscript received April 29, 2017; accepted May 8, 2017.

Address correspondence and reprint requests to James P. O'Callaghan, Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, 1095 Willowdale Road, Mailstop L-3014, Morgantown, WV 26505, USA. E-mail: jdo5@cdc.gov

Abbreviations used: Ach, acetylcholine; AChE, acetylcholinesterase; BBB, blood-brain barrier; CCL2, (C–C) chemokine ligand 2; CNS, central nervous system; CORT, corticosterone; CPF, chlorpyrifos; CPO, chlorpyrifos-oxon; DFP, diisopropyl fluorophosphate; GFAP, glial fibrillary acidic protein; GWI, Gulf War Illness; IL-1β, interleukin 1 beta; IL-6, interleukin 6; JAK, Janus kinase; LIF, leukemia inhibitory factor; OP, organophosphate; OSM, oncostatin M; PB, pyridostigmine bromide; PHY, physostigmine; $pSTAT3^{Tyr705}$, phosphorylated STAT3 tyrosine 705; SDS, sodium dodecyl sulfate; STAT3, signal transducer and activator of transcription 3; TNFα, tumor necrosis factor-alpha.

Research Advisory Committee (RAC) on Gulf War Veterans' Illnesses 2013; Heng 2016; White *et al.* 2016). The set of symptoms that characterizes GWI closely resembles the symptoms of protracted 'sickness behavior' (e.g., fatigue, nausea, sleep disturbances, cognitive impairments), a condition that is accompanied by a robust neuroinflammatory response seen in both humans and animal models (Dantzer *et al.* 2008; O'Callaghan *et al.* 2015). While a chronic or heightened neuroinflammatory response is linked to the symptoms of GWI in veterans, the underlying causes of this response have not been fully elucidated.

Soldiers that served in the Gulf War were exposed to a number of acetylcholinesterase (AChE) inhibitors. The irreversible AChE inhibitor, chlorpyrifos (CPF), was sprayed on uniforms and used in living quarters as an insecticide (Research Advisory Committee (RAC) on Gulf War Veterans' Illnesses 2008); another irreversible AChE inhibitor, dichlorvos, was used in 'pest strips' placed in encampments (Research Advisory Committee (RAC) on Gulf War Veterans' Illnesses 2008). Soldiers also were potentially exposed to another irreversible AChE inhibitor, the nerve agent, sarin, likely from a downwind plume following the demolition of munitions at several sites, notably, the Khamisiyah Ammunition Storage Facility (Research Advisory Committee (RAC) on Gulf War Veterans' Illnesses 2008; White *et al.* 2016). Finally, soldiers self-administered the reversible AChE inhibitor, pyridostigmine bromide (PB), which was consumed as a prophylactic treatment against potential nerve agent exposure (Tuovinen *et al.* 1999; Research Advisory Committee (RAC) on Gulf War Veterans' Illnesses 2008; White *et al.* 2016). The shared actions of these irreversible and reversible AChE inhibitors suggests that exposure to these compounds in theater may have contributed to the symptoms of Gulf War Illness (Fukuda *et al.* 1998; Cao *et al.* 2011; Patocka *et al.* 2015), specifically by increasing acetylcholine (ACh) as a result of the inhibition of AChE (Friedman *et al.* 1996; Golomb 2008).

While inhibition of AChE by GWI-relevant compounds serves as an attractive hypothesis for a contributory role of ACh in the development of GWI, other observations would argue against such a role. For example, AChE inhibition and the resulting increase in ACh levels should make these compounds anti-inflammatory agents caused by activation of the 'cholinergic anti-inflammatory pathway' (Pavlov *et al.* 2003; Pavlov and Tracey 2005), that is, effects that contrast with the known proinflammatory actions observed for organophosphates (OPs) in mouse and rat models (Spradling *et al.* 2011; O'Callaghan *et al.* 2015). The disparate potential roles for ACh in neuroinflammation make it seem possible that cholinergic mechanisms, and inhibition of AChE in particular, may not be responsible for symptoms associated with GWI. One way to address the role of AChE inhibition in GWI would be to assay the activity of AChE and the expression of proinflammatory mediators in samples obtained from animals exposed to GWI-relevant compounds and conditions. This approach would allow for a comparison among irreversible and reversible AChE inhibitors implicated in GWI with respect to their ability to cause neuroinflammation and inhibition of AChE.

Previously, we developed a mouse model of GWI that utilizes diisopropyl fluorophosphate (DFP) as a sarin surrogate and exogenous corticosterone (CORT) at levels associated with high physiological stress (Sapolsky *et al.* 1985) to replicate GW theater conditions (O'Callaghan *et al.* 2015). We found that exposure to DFP, an irreversible inhibitor of AChE, results in a brain-wide neuroinflammation that, paradoxically, is markedly augmented by prior exposure to the anti-inflammatory glucocorticoid, CORT. Here, we tested the hypothesis that AChE inhibition was not required for expression of neuroinflammatory mediators using our previously developed mouse model of GWI (O'Callaghan *et al.* 2015). We found that irreversible, but not reversible inhibition of AChE, was associated with neuroinflammation, effects enhanced by prior exposure to high physiological levels of CORT. Consistent with these findings, the downstream signaling effector of neuroinflammation, phosphorylated signal transducer and activator of transcription 3 tyrosine 705 ($pSTAT3^{Tyr705}$) (O'Callaghan *et al.* 2014) was activated by irreversible, but not reversible, inhibitors of AChE and was also enhanced by prior exposure to CORT. These findings indicate that the CORT-primed neuroinflammation associated with GWI-related AChE inhibitors is unlikely to be directly induced by AChE inhibition.

Methods

Materials

Drugs and chemicals were obtained from the following sources: chlorpyrifos oxon (CPO; Chem Service, Inc., West Chester, PA, USA), DFP (Sigma-Aldrich Co., St. Louis, MO, USA), PB (Sigma-Aldrich Co.), physostigmine (PHY; Sigma-Aldrich Co.), ethanol (Sigma-Aldrich Co.), CORT (Steraloids Inc., Newport, RI, USA), 5,5-dithio-bis-(2-nitrobenzoic acid) (Sigma-Aldrich Co.), tetraisopropyl pyrophosphoramide (Sigma-Aldrich Co.), and acetylthiocholine iodide (Sigma-Aldrich Co.). Rabbit Anti-phospho $STAT3^{tyr705}$ antibodies were obtained from Cell Signaling, Inc. (RRID: AB_621843; Beverly, MA, USA). The materials used in glial fibrillary acidic protein (GFAP) ELISA previously have been described in detail (O'Callaghan *et al.* 1991; O'Callaghan 2002). Material used for additional tissue analyses were of at least analytical grade and purchased from various commercial sources.

Animals

Adult male (8–12 weeks of age; weighing approximately 22 g) C57BL/6J mice were purchased from Jackson Labs (RRID: IMSR_JAX:000664; Bar Harbor, ME, USA). Upon arrival, mice were individually housed in a temperature- (21 ± 1°C) and humidity-controlled (50 ± 10%) colony room that was maintained under filtered positive-pressure ventilation on a 12 h light (0600 EDT)/12 h dark cycle (1800 EDT). The plastic cages were 46 cm in

length by 25 cm in width by 15 cm in height; cage bedding consisted of heat-treated pine shavings spread at a depth of 4 cm. Mice were single housed and given *ad libitum* access to food (Harlan 7913 irradiated NIH-31 modified 6% rodent chow) and water. All mouse procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee of the Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, and the animal colony was certified by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Dosing

Mice (n = at least four/group, animals were arbitrarily assigned to groups by the experimenter) were given intraperitoneal (i.p.) injections of saline (0.9%), peanut oil (CPO vehicle), CPO (8 mg/kg,) DFP (4 mg/kg), PB (3 mg/kg) or PHY (0.5 mg/kg) in the morning and returned to their home cage (experimenter was not blinded). The doses of AChE inhibitors were selected based on their ability to produce the symptoms of cholinergic crisis [e.g., SLUD (salivation, lacrimation, urination, and defecation) and seizures] while displaying mortality below LD25. CORT was given in the drinking water (400 mg/L in 1.2% EtOH) for 4 days prior to AChE inhibitor or vehicle exposure. This regimen of CORT was chosen because of its ability to achieve high circulating levels of this hormone, similar to those achieved with repeated stress (Ganon and McEwen 1990), and because it was capable of producing significant immunosuppression as evidenced by involution of the thymus (O'Callaghan *et al.* 1991). Here, average thymus weights were significantly reduced from 45.4 ± 2.7 mg in vehicle-treated to 12.8 ± 1.8 mg in CORT-treated mice with average body weight of 21.5 ± 0.4 g. This study used twice the CORT concentration in the drinking water, but employed a 4-day instead of a 7-day CORT exposure regimen in comparison to the CORT regimen used in our original mouse model of GWI (O'Callaghan *et al.* 2015). Mice were killed by decapitation at 30 min (for AChE activity assay) or 6 h (for all other analyses) post-AChE or vehicle injection. No differences were seen for the endpoints evaluated between mice exposed to peanut oil and saline (data not shown); therefore, peanut oil alone groups were excluded from analyses.

Brain dissection and preparation

Immediately after decapitation, whole brains were removed from the skull and the hippocampus and cortex were dissected free-hand on a thermoelectric cold plate (Model TCP-2; Aldrich Chemical Co., Milwaukee, WI, USA) using fine curved forceps (Roboz, Washington, DC, USA). Brain regions from one side of the brain were frozen and stored at −80°C until subsequent isolation of total RNA. Brain regions from the other side of the brain were weighed and then homogenized with the aid of a sonic probe (model XL-2005; Heat Systems, Farmingdale, NY, USA) in 10 volumes of hot (90–95°C) 1% sodium dodecyl sulfate (SDS). This tissue was then stored at −80°C until total protein assay and immunoassays of GFAP and immunoblots of $pSTAT3^{tyr705}$ were conducted. For the AChE activity assay, brains were bifurcated and one half of the brain was frozen and stored at −80°C until analysis.

RNA isolation, cDNA synthesis, and qPCR

The total RNA from the hippocampus and cortex were isolated using Trizol® reagent (Thermo Fisher Scientific, Waltham, MA, USA) and Phase-lock heavy gel (Eppendorf, AG Hamburg, Germany), and purified using RNeasy mini-spin columns (Qiagen, Valencia, CA, USA). Total RNA (1 μg) was reverse transcribed to cDNA using Superscript III and oligo $(dT)_{12–18}$ primers (Thermo Fisher Scientific) in a 20 μL reaction. Real-time PCR analysis of glyceraldehyde-3-phosphate dehydrogenase (endogenous control), tumor necrosis factor-alpha (TNFα), C–C chemokine ligand 2, leukemia inhibitor factor, interleukin 6 (IL-6), interleukin 1beta (IL-1β), oncostatin M and GFAP was performed using an Applied Biosystems 7500 real-time PCR system (Thermo Fisher Scientific) in combination with TaqMan® chemistry. All PCR amplifications (40 cycles) were performed in a total volume of 50 μL, containing 1 μL cDNA, 2.5 μL of the specific Assay of Demand primer/probe mix (Thermo Fisher Scientific), and 25 μL of Taqman® Universal master mix (Thermo Fisher Scientific). Sequence detection software (version 1.7; Applied Biosystems/Thermo Fisher Scientific) results were exported into Excel for further analysis. Relative quantification of gene expression was performed using the comparative threshold ($\Delta\Delta C_T$) method. Changes in mRNA expression levels were calculated after normalization to glyceraldehyde-3-phosphate dehydrogenase. The ratios obtained after normalization are expressed as fold changes over corresponding controls.

pSTAT3 immunoblot analysis

Activation of the Janus kinase (JAK)-STAT3 neuroinflammation effector pathway (O'Callaghan *et al.* 2014) was assessed by quantifying $pSTAT3^{tyr705}$ from immunoblots of tissue homogenates, with detection of fluorescent signals using an infrared fluorescence scanner (Licor Biosciences; Lincoln, NE, USA) as previously described (Sriram *et al.* 2004; Dinapoli *et al.* 2010; O'Callaghan *et al.* 2014). Briefly, following incubation with primary antibodies (rabbit anti-phospho-$STAT3^{tyr705}$[1 : 500]; RRID: AB_331586; Cell Signaling, Danvers, MA, USA), blots were washed with phosphate buffered saline with 0.1% Tween-20 and incubated with anti-rabbit fluorescent-labeled secondary antibody (1 : 2500; RRID: AB_621843) for 1 h prior to scanning by Licor. We note the general requirement for using focused microwave irradiation sacrifice to preserve steady-state *in vivo* phosphorylation does not apply in the case of $pSTAT3^{tyr705}$ (O'Callaghan and Sriram 2004).

Immunoassay of GFAP

GFAP was assayed in accordance with a previously described procedure (O'Callaghan 1991, 2002). In brief, a rabbit polyclonal antibody to GFAP (1 : 400; RRID: AB_10013382; DAKO, Carpenteria, CA, USA) was coated on the wells of Immulon-2 microtiter plates (Thermo Labsystems, Franklin, MA, USA). The SDS homogenates and standards were diluted in phosphate-buffered saline (pH 7.4) containing 0.5% Triton X-100. Standards consisted of SDS homogenates of hippocampus with known concentration of GFAP and were prepared the same way as the samples. After blocking non-specific binding with 5% non-fat dairy milk, aliquots of the homogenate and standards were added to the wells and incubated. Following washes, a mouse monoclonal antibody to GFAP (1 : 250; RRID: AB_477010; Sigma-Aldrich Co.) was added to 'sandwich' the GFAP between the two antibodies. An alkaline phosphatase-conjugated antibody directed against mouse IgG (1 : 2000; RRID: AB_2340075; Jackson ImmunoResearch Labs, West Grove, PA, USA) was then added and a colored reaction

product was obtained by subsequent addition of the enzyme substrate, p-nitrophenol. Quantification was achieved by spectrophotometry of the colored reaction product at 405 nm in a microplate reader, Spectra Max Plus, and analyzed using Soft Max Pro Plus software (Molecular Devices, Sunnyvale, CA, USA). The amount of GFAP in the samples was calculated as micrograms of GFAP per milligram total protein.

Acetylcholinesterase activity

Acetylcholinesterase activity was assessed via a protocol adapted from the Ellman method (Ellman *et al.* 1961; Lein and Fryer 2005). Briefly, one frozen cerebral hemisphere was homogenized with a sonic probe (mode XL-2005; Heat Systems) in 10 volumes of sodium phosphate buffer (0.1 M, pH 8.0) with 1% Triton X-100. Immediately following homogenization, the brains were centrifuged at 13 400 × g and the supernatant was removed and diluted 1 : 10 with sodium phosphate buffer prior to analysis. Following addition of a 5,5-dithio-bis-(2-nitrobenzoic acid)/tetraisopropyl pyrophosphoramide solution, samples were incubated for 5 min and reaction began when acetylthiocholine iodide was added. Quantification was achieved by spectrophotometry of the colored reaction product at 405 nm over a 10 min kinetic assay (16 cycles) in a Spectra Max Plus microplate reader and analyzed using Soft Max Pro Plus software (Molecular Devices). Acetylcholinesterase activity (μM substrate formed/min/mg total protein) was calculated based on the amount of total protein determined using the Pierce™ BCA Protein Assay kit (Thermo Fisher Scientific), per manufacturer's instructions. Acetylcholinesterase activity was normalized to saline control and is reported as a percentage.

Statistics

For calculation of sample size, ANOVA power analysis was performed using SigmaPlot (Systat Software, Inc., San Jose, CA, USA; RRID: SCR_003210; v12.5) using previously obtained mean differences and standard deviations between treated and control tissue with a power of 0.8 and $\alpha = 0.05$; the sample size was estimated at four mice per group. Larger sample sizes of 5–7 were utilized to control for AChE inhibition induced mortality and endpoint variability [removal of outliers via Grubbs' test ($\alpha = 0.05$)] to achieve final sample sizes of at least $n = 4$ per group. All statistical analyses were performed using SigmaPlot. Two-way ANOVAS were conducted on log transformed values with Fisher least significant difference *post-hoc* tests. Statistical significance was set at $\alpha = 0.05$ ($p < 0.05$), and all graphs show mean ± SEM of raw values, unless otherwise stated.

Results

Irreversible AChE inhibitors produce neuroinflammation that is markedly enhanced by CORT pretreatment

Administration of the irreversible AChE inhibitor, DFP (used as a sarin surrogate), resulted in an increased expression of CCL2 and TNFα in cortex and or hippocampus (Fig. 1). Prior treatment with CORT in the drinking water for 4 days resulted in significant increases in all six cytokines/chemokines in both brain regions (Fig. 1) (with the exception of IL-6 in hippocampus). Small increases (TNFα) or decreases (IL-6) also were observed for the CORT alone condition in cortex. The findings for DFP were extended to another irreversible inhibitor of AChE, CPO (the oxon metabolite of the insecticide CPF). Like DFP, administration of CPO alone produced a significant increase in the expression of some proinflammatory mediators in cortex and hippocampus (Fig. 2) (and a small decrease in expression of IL-6 in hippocampus). Also consistent with the data for DFP, CPO-induced neuroinflammation was markedly increased by prior treatment with CORT, with the exception of mRNA for IL-6 (Fig. 2). These results act to expand our DFP-based GWI model (O'Callaghan *et al.* 2015) to include another GW-relevant exposure, chlorpyrifos.

Reversible AChE inhibition does not produce neuroinflammation

Epidemiologic studies have associated the reversible AChE inhibitor, PB, a drug given as a prophylactic measure against nerve agent exposure during the Gulf War, with the symptomology of GWI (Steele *et al.* 2012; White *et al.* 2016). PB does not readily cross the blood-brain barrier (BBB) (Rice *et al.* 1997; Tuovinen *et al.* 1999; Song *et al.* 2002; Amourette *et al.* 2009); therefore, to assess the potential for reversible AChE inhibition in the central nervous system (CNS) to produce neuroinflammation, mice were exposed to PB or the CNS-penetrant reversible AChE inhibitor, PHY, with or without CORT pretreatment. In general, neither agent produced significant neuroinflammation alone or with prior CORT treatment, except for a minor effect on TNFα (Figs 3 and 4). In addition, reversible AChE inhibitors tend to be anti-inflammatory, reducing neuroinflammation significantly below control levels in some cases (Figs 3 and 4).

CORT pretreatment of irreversible (CPO and DFP) but not reversible (PHY and PB) AChE inhibitors produces STAT3 Activation

Proinflammatory cytokine signaling can activate the JAK/STAT3 pathway, as evidenced by the $pSTAT3^{tyr705}$ (O'Callaghan *et al.* 2014). Here, we observed an increase in $pSTAT3^{Tyr705}$ in the cortex and hippocampus of CORT+DFP and CORT+CPO exposed mice, an effect not seen following CORT+PB or CORT+PHY exposure (Fig. 5). This observation is consistent with the enhanced neuroinflammation induced by DFP and CPO following CORT pretreatment (Figs 1 and 2). Activation of the STAT3 pathway in the absence of astrogliosis, as is the case for exposure to DFP and CPO in our model (see below and O'Callaghan *et al.* 2015), is suggestive of the activation of microglia (O'Callaghan *et al.* 2014).

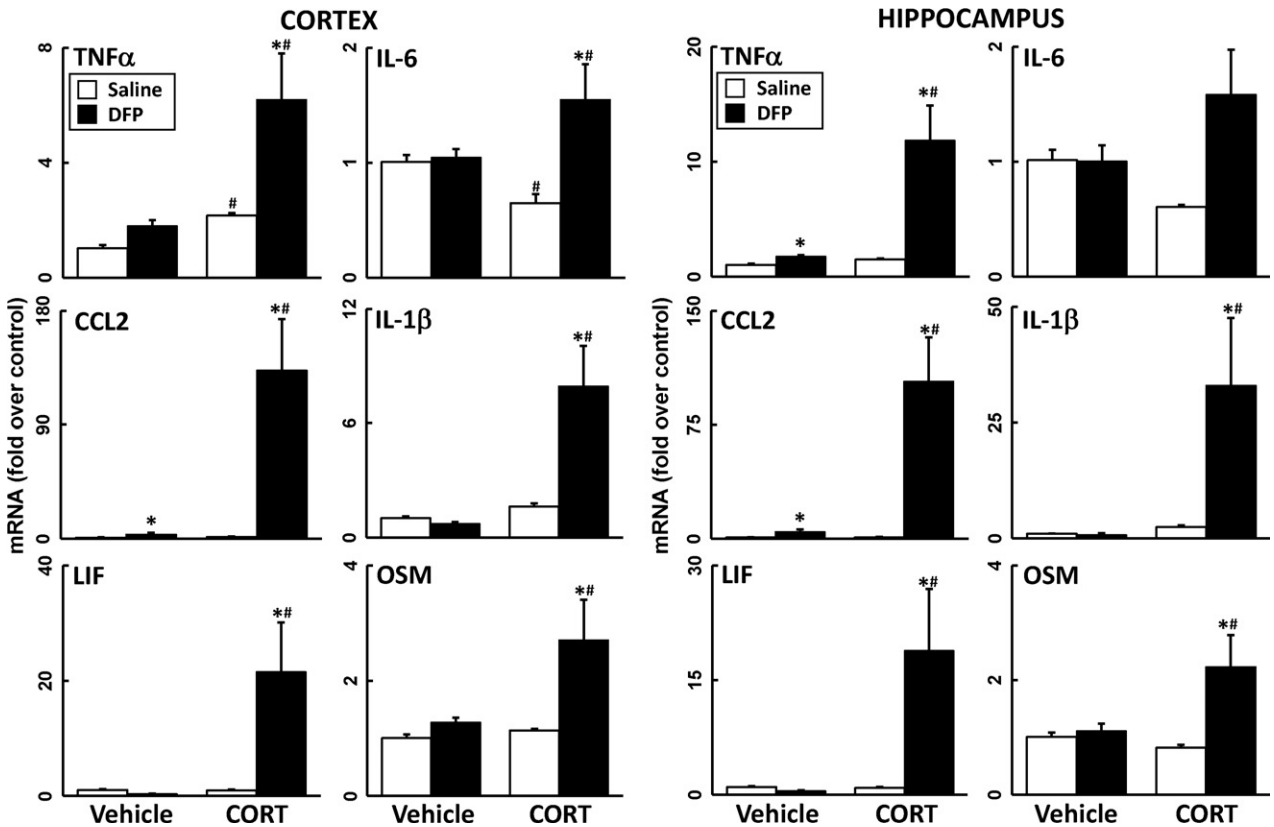

Fig. 1 Corticosterone (CORT) pretreatment exacerbates diisopropyl fluorophosphate (DFP)-induced neuroinflammation. Effects of DFP exposure (4 mg/kg, i.p.) with and without prior CORT treatment (400 mg/L, 1.2% EtOH) on neuroinflammation as measured by qPCR of inflammatory cytokines and chemokines at 6 h post-DFP. Tumor necrosis factor-alpha (TNFα), IL-6, (C–C) chemokine ligand 2 (CCL2), IL-1b, leukemia inhibitory factor (LIF), and oncostatin M (OSM) were measured in cortex (left panels) and hippocampus (right panels). Data represents mean ± SEM (N = 4–6 mice/group). Statistical significance of at least $p \leq 0.05$ is denoted by * compared to relevant control (vehicle or CORT) and # compared within treatment (saline or DFP).

DFP, CPO and PHY, but not PB, inhibit brain acetylcholinesterase activity, but prior CORT pretreatment reduces the inhibition caused by DFP and CPO

One hypothesis regarding AChE inhibitors and the development of GWI is that cholinergic effects of these compounds have made lasting physiological impacts that may contribute to the underlying cause of GWI (Golomb 2008). To evaluate this theory, the AChE enzyme activity was measured in mice treated with both irreversible and reversible AChE inhibitors with or without prior CORT treatment. As expected, DFP, CPO, and PHY exposure resulted in significant inhibition of enzyme activity 30 min after treatment and PB had no effect on AChE activity in the brain (Fig. 6). Interestingly, pretreatment with CORT significantly 'recovered' some of the AChE activity inhibited by DFP and CPO (Fig. 6). However, CORT pretreatment did not ameliorate PHY-induced AChE inhibition (Fig. 6). Furthermore, despite claims that stressors or stress hormone may increase BBB permeability to PB (Friedman *et al.* 1996; Hanin 1996; Shen 1998), there was no effect of PB on AChE activity in the brain with or without prior CORT exposure (Fig. 6). The recovery of AChE activity with DFP and CPO following CORT pretreatment suggests that the enhanced neuroinflammation seen with these conditions is not dependent on a particular degree of AChE inhibition. Moreover, CORT treatment prior to DFP or CPO exposure brings AChE activity back to a level comparable with PHY exposure, which we have shown does not instigate neuroinflammation (Fig. 4).

GFAP mRNA and protein levels were unchanged as a result of any AChE inhibitor exposure with or without CORT pretreatment

Damage to the CNS by any type of insult, including neurotoxicants, results in hypertrophy of astrocytes at sites of injury (O'Callaghan and Sriram 2005; O'Callaghan *et al.* 2008). Injury-induced activation of astrocytes is associated with an accumulation of the astrocyte intermediate filament protein, GFAP (O'Callaghan and Sriram 2005; O'Callaghan *et al.* 2014). Thus, GFAP expression and levels can be used

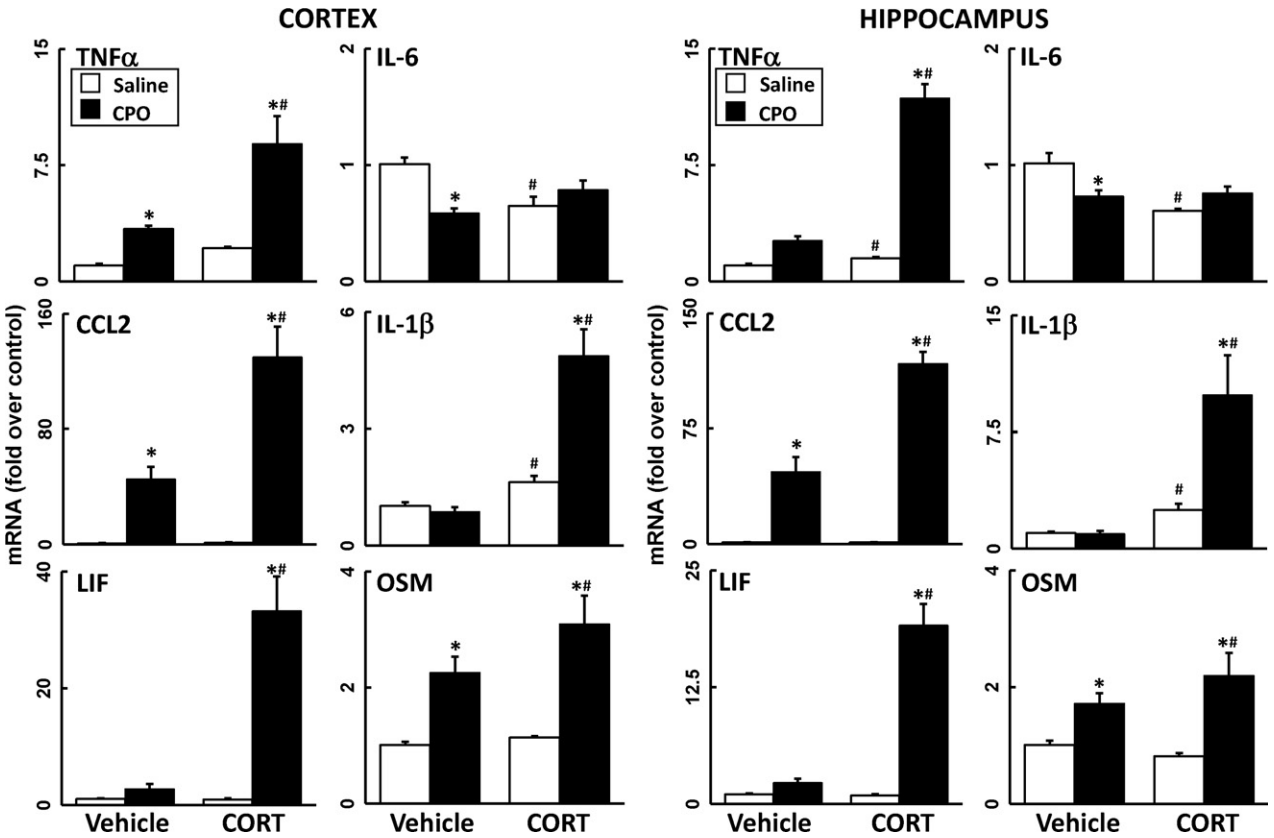

Fig. 2 Corticosterone (CORT) pretreatment exacerbates chlorpyrifos oxon (CPO)-induced neuroinflammation. Effects of CPO exposure (8 mg/kg, i.p.) with and without prior CORT treatment (400 mg/L, 1.2% EtOH) on neuroinflammation as measured by qPCR of inflammatory cytokines and chemokines at 6 h post-CPO. Tumor necrosis factor-alpha (TNFα), IL-6, (C–C) chemokine ligand 2 (CCL2), IL-1b, leukemia inhibitory factor (LIF), and oncostatin M (OSM) were measured in cortex (left panels) and hippocampus (right panels). Data represents mean ± SEM (N = 4–6 mice/group). Statistical significance of at least $p \leq 0.05$ is denoted by * compared with relevant control (vehicle or CORT) and # compared with treatment (saline or CPO).

as a biomarker of underlying CNS damage. With minor exceptions, no changes in GFAP or GFAP mRNA were seen with any exposure condition (data not shown), results consistent with our prior findings for DFP with and without CORT (O'Callaghan *et al.* 2015), and findings suggestive of a lack of underlying damage as a result of any treatment.

Discussion

GWI is a multisymptom disorder with characteristics of sickness behavior, a symptom profile known to be driven by underlying neuroinflammation (Steele 2000; Konsman *et al.* 2002; Dantzer *et al.* 2008; Bay-Richter *et al.* 2011). Among the exposures implicated in the etiology of GWI are AChE inhibitors: the nerve agent, sarin, the insecticide, CPF, and the nerve agent prophylactic, PB. Here, we have shown that exposure to the irreversible AChE inhibitors, DFP (a sarin surrogate) and CPO (the oxon derivative of CPF), results in neuroinflammatory responses in both the cortex and hippocampus that are augmented by 4 days of prior exposure to CORT in the drinking water. The enhanced neuroinflammation prompted by prior CORT exposure was associated with a subsequent increase in activation of STAT3, a key down-stream effector of neuroinflammation (O'Callaghan *et al.* 2014). The results obtained with DFP and CPO could not be extended to the reversible AChE inhibitors, PB and PHY. Moreover, the neuroinflammatory responses associated with exposure to DFP and CPO did not correlate with AChE inhibition, as the conditions that resulted in robust neuroinflammation (CORT+DFP and CORT+CPO) also blunted overall AChE inhibition. These observations suggest that the neuroinflammatory responses that may underlie the symptoms of GWI are not related to the cholinergic effects of these compounds in the CNS, as they are not generalizable across both irreversible and reversible inhibitors. However, neuroinflammation was instigated by both irreversible AChE inhibitors (DFP and CPO) that are also classified as OP compounds, suggesting that it may be the phosphorylating action of these chemicals that is the effector of GWI.

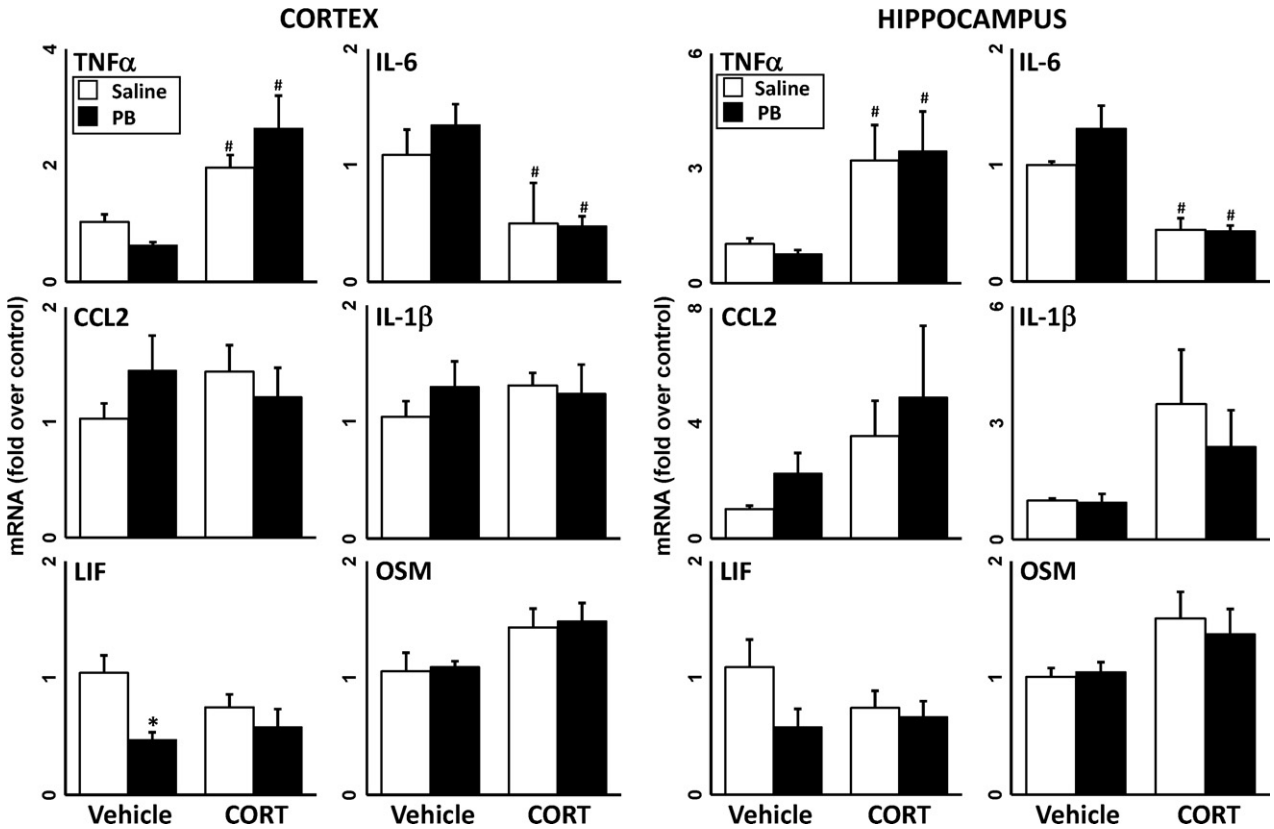

Fig. 3 Pyridostigmine bromide (PB) does not cause significant neuroinflammation with or without prior corticosterone (CORT) exposure. Effects of PB exposure (3 mg/kg, i.p.) with and without prior CORT treatment (400 mg/L, 1.2% EtOH) on neuroinflammation as measured by qPCR of inflammatory cytokines and chemokines at 6 h post-PB. Tumor necrosis factor-alpha (TNFα), IL-6, (C–C) chemokine ligand 2 (CCL2), IL-1b, leukemia inhibitory factor (LIF), and oncostatin M (OSM) were measured in cortex (left panels) and hippocampus (right panels). Data represents mean ± SEM ($N = 4$–6 mice/group). Statistical significance of at least $p \leq 0.05$ is denoted by * compared with relevant control (vehicle or CORT) and # compared with treatment (saline or PB).

Our present findings extend our previously established GWI model (using DFP as a sarin surrogate) (O'Callaghan *et al.* 2015) to the GWI-relevant pesticide, CPF. The augmented neuroinflammatory response seen with CORT administration prior to both DFP and CPO is paradoxical, because glucocorticoids, such as CORT, traditionally are considered to be immunosuppressants (Barnes 2006; Coutinho and Chapman 2011). The mechanism through which this CORT-'primed' neuroinflammatory response is achieved is currently unknown, but our findings are consistent with other studies that have found similar pro-inflammatory effects with stressor or stress hormones alone or in response to neuroinflammatory exposures (Johnson *et al.* 2003; O'Connor *et al.* 2003; Loram *et al.* 2011). CORT priming, however, does not occur with all neuroinflammatory exposures. For example, prior CORT administration in the drinking water does not enhance the inflammatory response observed after dopaminergic neurotoxicity caused by MPTP, despite enhancing the neuroinflammatory response to the dopaminergic neurotoxicant, METH (Kelly *et al.* 2012).

AChE inhibition does not appear to drive neuroinflammation observed in our GWI model. The irreversible inhibitors of AChE, DFP and CPO, and the brain penetrant reversible inhibitor of AChE, PHY, inhibited brain AChE activity as expected. Such effects likely do not underlie neuroinflammation, because inhibition of AChE by the reversible AChE inhibitor, PHY, did not induce neuroinflammation with or without prior CORT. Moreover, CORT-enhanced neuroinflammation associated with exposure to DFP and CPO occurred despite a reduction in AChE inhibition by these compounds when given with CORT pretreatment. One of the theories regarding the initiation of GWI is that stressors precipitated adverse effects of PB, administered as a nerve agent prophylactic (Friedman *et al.* 1996; Research Advisory Committee (RAC) on Gulf War Veterans' Illnesses 2008), potentially by allowing this compound to gain entry to the CNS. PB has a quaternary amine structure that should prevent BBB penetration and limit inhibition of AChE activity to the periphery (Rice *et al.* 1997; Tuovinen *et al.* 1999; Song *et al.* 2002; Amourette *et al.* 2009). Exposure to

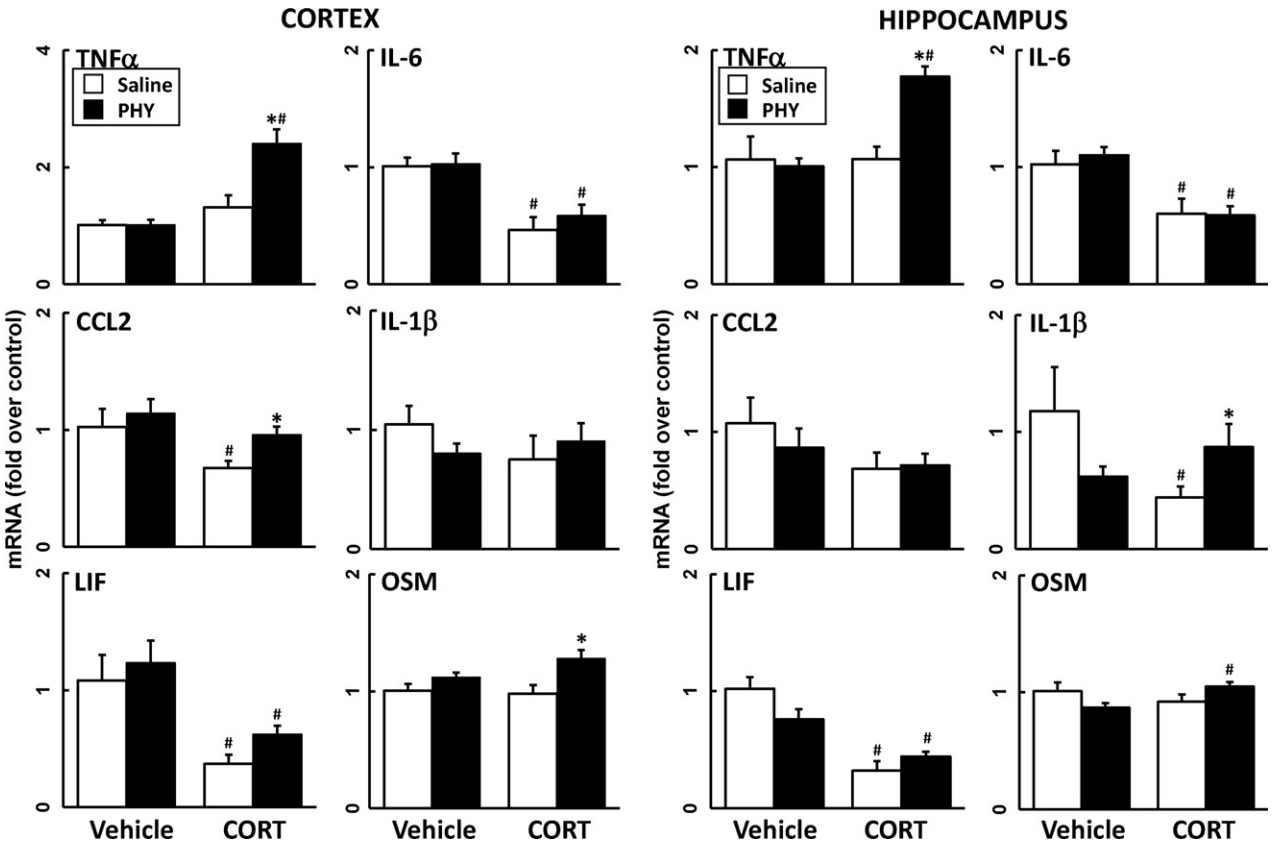

Fig. 4 The brain penetrant AChE inhibitior, physostigmine (PHY), has little effect on neuroinflammation in the presence of corticosterone (CORT) pretreatment. Effects of PHY exposure (0.5 mg/kg, i.p.) with and without prior CORT treatment (400 mg/L, 1.2% EtOH) on neuroinflammation as measured by qPCR of inflammatory cytokines and chemokines at 6 h post-PHY. Tumor necrosis factor-alpha (TNFα), IL-6, (C–C) chemokine ligand 2 (CCL2), IL-1b, leukemia inhibitory factor (LIF), and oncostatin M (OSM) were measured in cortex (left panels) and hippocampus (right panels). Data represents mean ± SEM (N = 4–6 mice/group). Statistical significance of at least $p \leq 0.05$ is denoted by * compared with relevant control (vehicle or CORT) and # compared with treatment (saline or PHY).

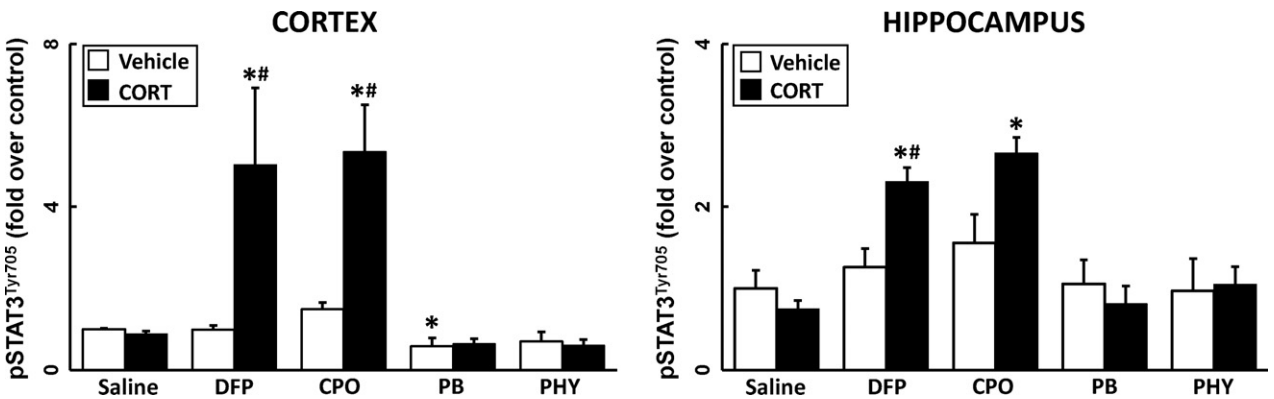

Fig. 5 Prior corticosterone (CORT) treatment significantly increases phosphorylated signal transducer and activator of transcription 3 tyrosine 705 (pSTAT3^{tyr705}) levels in diisopropyl fluorophosphate (DFP) and chlorpyrifos oxon (CPO) treated mice. Effects of CORT pretreatment (400 mg/L, 1.2% EtOH) on the phosphorylation of STAT3 at 6 h following AChE inhibitor exposure. pSTAT3^{tyr705} protein was measured in the cortex and hippocampus of saline, DFP, CPO, Pyridostigmine bromide (PB), and physostigmine (PHY) treated mice. Data represents mean ± SEM (N = 4–6 mice/group). Statistical significance of at least $p \leq 0.05$ is denoted by * compared with relevant control (vehicle or CORT) and # compared within treatment (saline or AChE inhibitor).

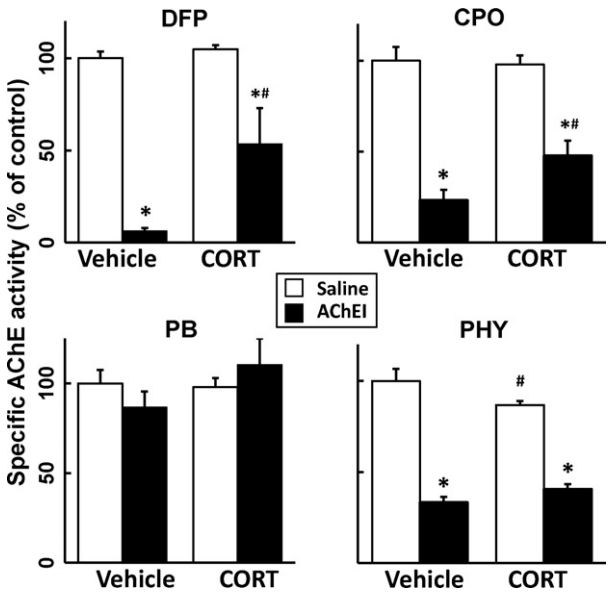

Fig. 6 Neuroactive AChE inhibitors significantly reduce AChE activity in the brain, an effect that is reversed by corticosterone (CORT) diisopropyl fluorophosphate (DFP) and CORT chlorpyrifos oxon (CPO). AChE activity was measured in one brain hemisphere 30 min following AChE inhibitor exposure [DFP, CPO, pyridostigmine bromide (PB), and physostigmine (PHY)] with or without CORT pretreatment (400 mg/L, 1.2% EtOH). Data represents mean % ± SEM ($N = 4$–6 mice/group). Statistical significance of at least $p \leq 0.05$ is denoted by * compared with relevant control (vehicle or CORT) and # compared with treatment (saline or AChE inhibitor).

acute stressors/stress hormones (e.g., cortisol), however, can increase permeability of the BBB (Friedman *et al.* 1996; Esposito *et al.* 2002). Thus, several studies have suggested that symptoms of GWI result from the actions of a peripherally-acting AChE inhibitor (i.e., PB) entering into the CNS, at least in part due to physiological stress-induced permeability of the BBB (Friedman *et al.* 1996; Hanin 1996; Shen 1998). Our findings suggest that any effects of PB related to GWI, in our model, are not because of brain entry of this compound resulting from pretreatment with CORT, because brain AChE activity was not reduced following exposure to these two treatments. These findings are consistent with those from other studies where PB permeability changes were not found following exposure to high physiological levels of CORT (Park *et al.* 2008; Amourette *et al.* 2009). Furthermore, recent evidence has indicated that exogenous glucocorticoid exposure actually may serve to strengthen the BBB, restricting, rather than facilitating the movement of peripherally administered chemicals and biologics into the brain (Calabria *et al.* 2006; Chen *et al.* 2013; Furihata *et al.* 2015).

Our animal model-based findings do more to rule out a role of AChE in GWI than they do to point to a specific non-AChE mechanism. Nevertheless, our findings provide indirect support to numerous prior investigations that point to: disrupted axonal transport via covalent binding of OPs to tubulin (Prendergast *et al.* 2007), disruption of neurofilament protein function by aberrant phosphorylation (Abou-Donia *et al.* 1988), reduced hippocampal neurogenesis (Parihar *et al.* 2013) and increases in amyloid precursor protein and Aβ levels (Sanchez-Santed *et al.* 2016), all as potential (non-AChE related) effects underlying symptoms exhibited by ill GW veterans. Beyond some of these 'alternative' targets of irreversible AChE inhibitors that have been implicated in GWI, many key pathways may contain additional vulnerable phospho-substrates that, upon phosphorylation by OPs, may alter signal transduction in a manner resulting in the symptoms of GWI. Screening tools for phosphoprofiling assessments (Zhu *et al.* 2010; Singec *et al.* 2016; Tinti *et al.* 2017) can be implemented to discover and characterize novel targets of organophosphorylation by OPs in animal models with the overall goal of identifying drug-able targets to treat GWI. While our present findings draw focus away from the AChE inhibiting activity of these compounds as a basis for the neuroinflammatory effects associated with GWI, our observations do not detract from a role of these AChE inhibiting exposures in GWI. Multiple epidemiological studies have implicated AChE inhibitors (pesticides, PB, nerve agent) in the etiology of symptoms associated with GWI (Research Advisory Committee (RAC) on Gulf War Veterans' Illnesses 2013). For example, a recent study (Steele *et al.* 2015) showed that butyrylcholinesterase genotypes possessed by the most ill GW veterans may have resulted in lower metabolism and higher exposure levels of self-administered PB. Such findings points to a role of genetic susceptibility in GWI and suggest the need to more broadly assess the potential diversity of targets underlying the disorder.

While neuroinflammatory responses often accompany neurotoxicant-induced neuronal damage (Whittington *et al.* 1989; O'Callaghan and Jensen 1992; Kelly *et al.* 2012; O'Callaghan *et al.* 2014), including exposure to irreversible AChE inhibitors (Chapman *et al.* 2006; Stapleton and Chan 2009; Li *et al.* 2011; Lim *et al.* 2011; Banks and Lein 2012; Ferchmin *et al.* 2014), neuroinflammation can occur in the absence of neuronal damage (O'Callaghan *et al.* 2014). Our present findings are consistent with the induction of neuroinflammation in the absence of damage, because GFAP mRNA and GFAP were not increased, as would have been expected if underlying neural damage had elicited astrogliosis, a dominant cellular reaction following exposure to broad classes of known neurotoxic conditions (O'Callaghan and Sriram 2005).

The observed activation of STAT3, a transcription factor associated with both damage-induced astrogliosis and neuroinflammation in the absence of neural damage and astrogliosis (O'Callaghan *et al.* 2014), was expected because CORT-primed DFP- and CPO-induced neuroinflammation,

even in the absence of neural damage, would signal through activation of STAT3. Because STAT3 was activated, it is unlikely that enhanced expression of neuroinflammatory mediators occurred in the absence of an increase in the mediators themselves, as opposed to their mRNA alone. We know this to be the case, because in the brain slice preparation, STAT3 activation during neuroinflammatory responses is enhanced by the addition of proinflammatory cytokines and is blocked by neutralizing antibodies to proinflammatory cytokines (Damiani and O'Callaghan 2007). While activated STAT3 is required for the induction of astrogliosis (Herrmann *et al.* 2008; O'Callaghan *et al.* 2014), activation of the STAT3 pathway also occurs in response to exposure to the known inflammagen, lipopolysaccharide, in the absence of astrogliosis, likely through activation of microglia (O'Callaghan *et al.* 2014). These observations suggest that the neuroinflammation-related activation of the STAT3 pathway after DFP and CPO results from inflammatory signaling through microglia.

Taken together, our findings show that exposure to CORT at levels associated with high physiological stress prior to acute administration of the GW-relevant chemicals, DFP and CPO, results in a robust neuroinflammatory response that can serve as the basis of sickness behavior-like symptoms associated with GWI. These effects are not related to the AChE inhibition induced by these agents. Both DFP and CPO, unlike PB and PHY, are OP compounds. It is possible that DFP and CPO exert their effects on the brain through the 'organophosphorylation' of certain neuroimmune targets, such as the JAK/STAT3 pathway or the phosphorylation and dysregulation of yet to be identified signaling pathways/substrates. While further research is needed to evaluate the role of phosphorylation in the neuroimmune system relevant to GWI, several studies have documented the role of protein phosphorylation in OP-induced neuropathy (Abou-Donia *et al.* 1988; Choudhary *et al.* 2001; Flaskos 2014). Thus, our findings may open a new avenue for discovery of therapeutic targets and treatments for GWI based on phosphoprotein profiling of signaling pathways (Zhu *et al.* 2010) involved in neuroimmune responses. Our data also demonstrate the potential for physiological stress to not only serve as a factor contributing to GWI, but also to play a role in exacerbation of chronic inflammatory disorders.

Acknowledgments and conflict of interest disclosure

We appreciate the excellent technical assistance provided by Brenda K. Billig and Christopher M. Felton. Funding for this project was supported by: Intramural funds from the Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health. Congressionally Directed Medical Research Programs: Gulf War Illness Research Program Grants (GW080150, GW120037, GW120045). The authors declare that they have no competing interests.

All experiments were conducted in compliance with the ARRIVE guidelines.

Disclaimer: The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

References

Abou-Donia M. B., Lapadula D. M. and Suwita E. (1988) Cytoskeletal proteins as targets for organophosphorus compound and aliphatic hexacarbon-induced neurotoxicity. *Toxicology* **49**, 469–477.

Amourette C., Lamproglou I., Barbier L., Fauquette W., Zoppe A., Viret R. and Diserbo M. (2009) Gulf War illness: effects of repeated stress and pyridostigmine treatment on blood-brain barrier permeability and cholinesterase activity in rat brain. *Behav. Brain Res.* **203**, 207–214.

Banks C. N. and Lein P. J. (2012) A review of experimental evidence linking neurotoxic organophosphorus compounds and inflammation. *Neurotoxicology* **33**, 575–584.

Barnes P. J. (2006) How corticosteroids control inflammation: quintiles Prize Lecture 2005. *Br. J. Pharmacol.* **148**, 245–254.

Bay-Richter C., Janelidze S., Hallberg L. and Brundin L. (2011) Changes in behaviour and cytokine expression upon a peripheral immune challenge. *Behav. Brain Res.* **222**, 193–199.

Calabria A. R., Weidenfeller C., Jones A. R., de Vries H. E. and Shusta E. V. (2006) Puromycin-purified rat brain microvascular endothelial cell cultures exhibit improved barrier properties in response to glucocorticoid induction. *J. Neurochem.* **97**, 922–933.

Cao J., Varnell A. and Cooper D. (2011) Gulf War Syndrome: a role for organophosphate induced plasticity of locus coeruleus neurons. *Nat. Preced.* doi:10.1038/npre.2011.6057.2.

Chapman S., Kadar T. and Gilat E. (2006) Seizure duration following sarin exposure affects neuro-inflammatory markers in the rat brain. *Neurotoxicology* **27**, 277–283.

Chen X., Zhao Z., Chai Y., Luo L., Jiang R., Dong J. and Zhang J. (2013) Stress-does hydrocortisone reduces critical illness-related corticosteroid insufficiency associated with severe traumatic brain injury in rats. *Crit. Care* **17**, R241.

Choudhary S., Joshi K. and Gill K. D. (2001) Possible role of enhanced microtubule phosphorylation in dichlorvos induced delayed neurotoxicity in rat. *Brain Res.* **897**, 60–70.

Coutinho A. E. and Chapman K. E. (2011) The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. *Mol. Cell. Endocrinol.* **335**, 2–13.

Damiani C. L. and O'Callaghan J. P. (2007) Recapitulation of cell signaling events associated with astrogliosis using the brain slice preparation. *J. Neurochem.* **100**, 720–726.

Dantzer R., O'Connor J. C., Freund G. G., Johnson R. W. and Kelley K. W. (2008) From inflammation to sickness and depression: when the immune system subjugates the brain. *Nat. Rev. Neurosci.* **9**, 46–56.

Dinapoli V. A., Benkovic S. A., Li X., Kelly K. A., Miller D. B., Rosen C. L., Huber J. D. and O'Callaghan J. P. (2010) Age exaggerates proinflammatory cytokine signaling and truncates signal transducers and activators of transcription 3 signaling following ischemic stroke in the rat. *Neuroscience* **170**, 633–644.

Ellman G. L., Courtney K. D., Andres V., Francisco S. and Featherstone R. M. (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7**, 88–95.

Esposito P., Chandler N., Kandere K., Basu S., Jacobson S., Connolly R., Tutor D. and Theoharides T. C. (2002) Corticotropin-releasing hormone and brain mast cells regulate blood-brain-barrier permeability induced by acute stress. *J. Pharmacol. Exp. Ther.* **303**, 1061–1066.

Ferchmin P. A., Andino M., Reyes Salaman R. *et al.* (2014) 4R-cembranoid protects against diisopropylfluorophosphate-mediated neurodegeneration. *Neurotoxicology* **44**, 80–90.

Flaskos J. (2014) The neuronal cytoskeleton as a potential target in the developmental neurotoxicity of organophosphorothionate insecticides. *Basic Clin. Pharmacol. Toxicol.* **115**, 201–208.

Friedman A., Kaufer D., Shemer J., Hendler I., Soreq H. and Tur-Kaspa I. (1996) Pyridostigmine brain penetration under stress enhances neuronal excitability and induces early immediate transcriptional response. *Nat. Med.* **2**, 1382–1385.

Fukuda K., Nisenbaum R., Stewart G. *et al.* (1998) Chronic multisymptom illness affecting Air Force veterans of the Gulf War. *JAMA* **280**, 981–988.

Furihata T., Kawamatsu S., Ito R., Saito K., Suzuki S., Kishida S., Saito Y., Kamiichi A. and Chiba K. (2015) Hydrocortisone enhances the barrier properties of HBMEC/ciβ, a brain microvasuclar endothelial cell line, through mesenchymal-to-endothelial transition-like effects. *Fluids Barriers CNS* **12**, 7.

Ganon M. N. and McEwen B. S. (1990) Calmodulin involvement in stress- and corticosterone-induced down-regulation of cyclic AMP-generating systems in brain. *J. Neurochem.* **55**, 276–284.

Golomb B. A. (2008) Acetylcholinesterase inhibitors and Gulf War illnesses. *Proc. Natl Acad. Sci. USA* **105**, 4295–4300.

Hanin I. (1996) The Gulf War, stress and a leaky blood-brain barrier. *Nat. Med.* **2**, 1307–1308.

Heng H. H. Q. (2016) Challenges and new strategies for Gulf War Illness research. *Environ. Dis.* **1**, 118–125.

Herrmann J. E., Imura T., Song B., Qi J., Ao Y., Nguyen T. K., Korsak R. A., Takeda K., Akira S. and Sofroniew M. V. (2008) STAT3 is a critical regulator of astrogliosis and scar formation after spinal cord injury. *J. Neurosci.* **28**, 7231–7243.

Johnson J. D., O'Connor K. A., Hansen M. K., Watkins L. R. and Maier S. F. (2003) Effects of prior stress on LPS-induced cytokine and sickness responses. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **284**, R422–R432.

Kelly K. A., Miller D. B., Bowyer J. F. and O'Callaghan J. P. (2012) Chronic exposure to corticosterone enhances the neuroinflammatory and neurotoxic responses to methamphetamine. *J. Neurochem.* **122**, 995–1009.

Konsman J. P., Parnet P. and Dantzer R. (2002) Cytokine-induced sickness behaviour: mechanisms and implications. *Trends Neurosci.* **25**, 154–159.

Lein P. J. and Fryer A. D. (2005) Organophosphorus insecticides induce airway hyperreactivity by decreasing neuronal M2 muscarinic receptor function independent of acetylcholinesterase inhibition. *Toxicol. Sci.* **83**, 166–176.

Li Y., Lein P. J., Liu C., Bruun D. A., Tewolde T., Ford G. and Ford B. D. (2011) Spatiotemporal pattern of neuronal injury induced by DFP in rats: a model for delayed neuronal cell death following acute OP intoxication. *Toxicol. Appl. Pharmacol.* **253**, 261–269.

Lim K. L., Tay A., Nadarajah V. D. and Mitra N. K. (2011) The effect of consequent exposure of stress and dermal application of low doses of chlorpyrifos on the expression of glial fibrillary acidic protein in the hippocampus of adult mice. *J. Occup. Med. Toxicol.* **6**, 4.

Loram L. C., Taylor F. R., Strand K. A., Frank M. G., Sholar P., Harrison J. A., Maier S. F. and Watkins L. R. (2011) Prior exposure to glucocorticoids potentiates lipopolysaccharide induced mechanical allodynia and spinal neuroinflammation. *Brain Behav. Immun.* **25**, 1408–1415.

O'Callaghan J. P. (1991) Quantification of glial fibrillary acidic protein: comparison of slot-immunobinding assays with a novel sandwich ELISA. *Neurotoxicol. Teratol.* **13**, 275–281.

O'Callaghan J. P. (2002) Measurement of glial fibrillary acidic protein. *Curr. Protoc. Toxicol.* **Chapter 12**, Unit 12.8.

O'Callaghan J. P. and Jensen K. F. (1992) Enhanced expression of glial fibrillary acidic protein and the cupric silver degeneration reaction can be used as sensitive and early indicators of neurotoxicity. *Neurotoxicology* **13**, 113–122.

O'Callaghan J. P. and Sriram K. (2004) Focused microwave irradiation of the brain preserves in vivo protein phosphorylation: comparison with other methods of sacrifice and analysis of multiple phosphoproteins. *J. Neurosci. Methods* **135**, 159–168.

O'Callaghan J. P. and Sriram K. (2005) Glial fibrillary acidic protein and related glial proteins as biomarkers of neurotoxicity. *Expert Opin. Drug Saf.* **4**, 433–442.

O'Callaghan J. P., Brinton R. E. and McEwen B. S. (1991) Glucocorticoids regulate the synthesis of glial fibrillary acidic protein in intact and adrenalectomized rats but do not affect its expression following brain injury. *J. Neurochem.* **57**, 860–869.

O'Callaghan J. P., Sriram K. and Miller D. B. (2008) Defining "neuroinflammation". *Ann. N. Y. Acad. Sci.* **1139**, 318–330.

O'Callaghan J. P., Kelly K. A., VanGilder R. L., Sofroniew M. V. and Miller D. B. (2014) Early activation of STAT3 regulates reactive astrogliosis induced by diverse forms of neurotoxicity. *PLoS ONE* **9**, e102003.

O'Callaghan J. P., Kelly K. A., Locker A. R., Miller D. B. and Lasley S. M. (2015) Corticosterone primes the neuroinflammatory response to DFP in mice: potential animal model of Gulf War Illness. *J. Neurochem.* **133**, 1–14.

O'Connor K. A., Johnson J. D., Hansen M. K., Wieseler Frank J. L., Maksimova E., Watkins L. R. and Maier S. F. (2003) Peripheral and central proinflammatory cytokine response to a severe acute stressor. *Brain Res.* **991**, 123–132.

Parihar M. L., Kumar A., Gamanagatti S., Bhalla A. S., Mishra B., Kumar S., Jama M. and Misra M. C. (2013) Role of splenic artery embolization in management of traumatic splenic injuries: a prospective study. *Indian J. Surg.* **75**, 361–367.

Park D., Jeon J. H., Shin S., Jang J. Y., Choi B., Nahm S.-S., Kang J.-K., Hwang S.-Y., Kim J.-C. and Kim Y.-B. (2008) Debilitating stresses do not increase blood–brain barrier permeability: lack of the involvement of corticosteroids. *Environ. Toxicol. Pharmacol.* **26**, 30–37.

Patocka J., Honegr J. and Soukup O. (2015) Gulf war syndrome – a syndrome or not? *Toxin. Rev.* **34**, 43–52.

Pavlov V. A. and Tracey K. J. (2005) The cholinergic anti-inflammatory pathway. *Brain Behav. Immun.* **19**, 493–499.

Pavlov V. A., Wang H., Czura C. J., Friedman S. G. and Tracey K. J. (2003) The cholinergic anti-inflammatory pathway: a missing link in neuroimmunomodulation. *Mol. Med.* **9**, 125–134.

Prendergast M. A., Self R. L., Smith K. J., Ghayoumi L., Mullins M. M., Butler T. R., Buccafusco J. J., Gearhart D. A. and Terry A. V., Jr (2007) Microtubule-assoicated targets in chlorpyrifos oxon hippocampal neurotoxicity. *J. Pharmacol. Exp. Ther.* **322**, 1117–1128.

Research Advisory Committee (RAC) on Gulf War Veterans' Illnesses (2008) *Gulf War Illness and the Health of Gulf War Veterans: scientific findings and recommendations*. US Government Printing Office, Washington, DC.

Research Advisory Committee (RAC) on Gulf War Veterans' Illnesses (2013) *Gulf War Illness and the Health of Gulf War Veterans: Research Update and Recommendations, 2009–2013*. US Government Printing Office, Washington, DC.

Rice P. J., Drewes C. D., Klubertanz T. M., Bradbury S. P. and Coats J. R. (1997) Acute toxicity and behavioral effects of chlorpyrifos, permethrin, phenol, strychnine, and 2,4-dinitrophenol to 30-day-old Japanese medaka (Oryzias latipes). *Environ. Toxicol. Chem.* **16**, 696–704.

Sanchez-Santed F., Colomina M. T. and Herrero Hernandez E. (2016) Organophosphate pesticide exposure and neurodegeneration. *Cortex* **74**, 417–426.

Sapolsky R. M., Krey L. C. and McEwen B. S. (1985) Prolonged glucocorticoid exposure reduces hippocampal neuron number: implications for aging. *J. Neurosci.* **5**, 1222–1227.

Shen Z. X. (1998) Pyridostigmine bromide and Gulf War syndrome. *Med. Hypotheses* **51**, 235–237.

Singec I., Crain A. M., Hou J. *et al.* (2016) Quantitative analysis of human pluripotency and neural specification by in-depth (Phospho) proteomic profiling. *Stem Cell Reports* **7**, 527–542.

Song X., Tian H., Bressler J., Pruett S. and Pope C. (2002) Acute and repeated restraint stress have little effect on pyridostigmine toxicity or brain regional cholinesterase inhibition in rats. *Toxicol. Sci.* **69**, 157–164.

Spradling K. D., Lumley L. A., Robison C. L., Meyerhoff J. L. and Dillman J. F., 3rd (2011) Transcriptional responses of the nerve agent-sensitive brain regions amygdala, hippocampus, piriform cortex, septum, and thalamus following exposure to the organophosphonate anticholinesterase sarin. *J. Neuroinflammation* **8**, 84.

Sriram K., Benkovic S. A., Hebert M. A., Miller D. B. and O'Callaghan J. P. (2004) Induction of gp130-related cytokines and activation of JAK2/STAT3 pathway in astrocytes precedes up-regulation of glial fibrillary acidic protein in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of neurodegeneration: key signaling pathway for ast. *J. Biol. Chem.* **279**, 19936–19947.

Stapleton A. R. and Chan V. T. (2009) Subtoxic chlorpyrifos treatment resulted in differential expression of genes implicated in neurological functions and development. *Arch Toxicol* **83**, 319–333.

Steele L. (2000) Prevalence and patterns of Gulf War illness in Kansas veterans: association of symptoms with characteristics of person, place, and time of military service. *Am. J. Epidemiol.* **152**, 992–1002.

Steele L., Sastre A., Gerkovich M. M. and Cook M. R. (2012) Complex factors in the etiology of Gulf War illness: wartime exposures and risk factors in veteran subgroups. *Environ. Health Perspect.* **120**, 112–118.

Steele L., Lockridge O., Gerkovich M. M., Cook M. R. and Sastre A. (2015) Butyrylcholinesterase genotype and enzyme activity in relation to gulf war illness: preliminary evidence of gene-exposure interaction from a case-control study of 1991 gulf war veterans. *Envrion. Health* **14**, 4.

Tinti M., Panni S. and Cesareni G. (2017) Profiling phosphopeptide-binding domain recognition specificity using peptide microarrays. *Methods Mol. Biol.* **1518**, 177–193.

Tuovinen K., Kaliste-Korhonen E., Raushel F. M. and Hänninen O. (1999) Success of pyridostigmine, physostigmine, eptastigmine and phosphotriesterase treatments in acute sarin intoxication. *Toxicology* **134**, 169–178.

White R. F., Steele L., O'Callaghan J. P. *et al.* (2016) Recent research on Gulf War illness and other health problems in veterans of the 1991 Gulf War: effects of toxicant exposures during deployment. *Cortex* **74**, 449–475.

Whittington D. L., Woodruff M. L. and Baisden R. H. (1989) The time-course of trimethyltin-induced fiber and terminal degeneration in hippocampus. *Neurotoxicol. Teratol.* **11**, 21–33.

Zhu H., O'Brien J. J., O'Callaghan J. P. *et al.* (2010) Nerve agent exposure elicits site-specific changes in protein phosphorylation in mouse brain. *Brain Res.* **1342**, 11–23.